(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number
WO 02/22861 A1

(51) International Patent Classification[7]: C12Q 1/26, 1/28, C12N 15/81, 9/02

(21) International Application Number: PCT/US00/28768

(22) International Filing Date: 13 October 2000 (13.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/661,093 13 September 2000 (13.09.2000) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/661,093 (CON)
Filed on 13 September 2000 (13.09.2000)

(71) Applicant *(for all designated States except US)*: **CALIFORNIA INSTITUTE OF TECHNOLOGY** [US/US]; 1200 East California Boulevard, Pasadena, CA 91225 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ARNOLD, Frances, H.** [US/US]; 629 S. Grand Avenue, Pasadena, CA 91105 (US). **JOO, Hyun** [KR/US]; Apartment #8, 533 S. Hudson Avenue, Pasadena, CA 91101 (US).

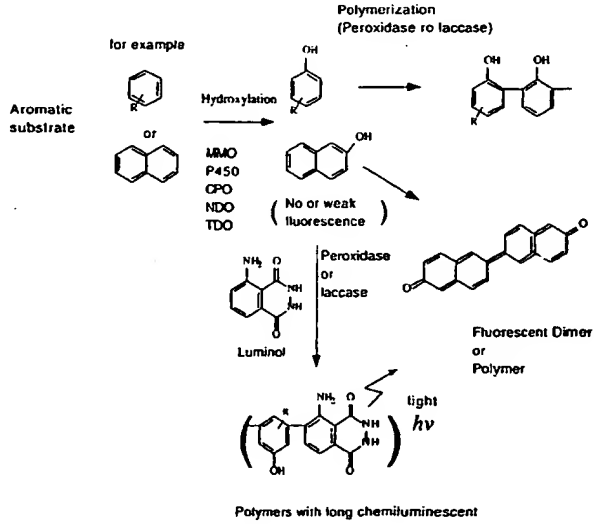
(74) Agents: **SCHAFFER, Robert** et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022-7513 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: OXYGENASE ENZYMES AND SCREENING METHOD



Polymers with long chemiluminescent

(57) Abstract: A method for detecting the presence of an oxygenated compound which is produced when a substrate is reacted with an oxygenase for the substrate. The method involves reacting a coupling enzyme with the oxygenated compound to form a polymeric oxygenated compound which is fluorescent or luminescent. Measurement of the fluorescence or luminescence of the polymeric oxygenated compound provides indirect detection of the oxygenated compound produced by reaction of the oxygenase with the substrate. The method is carried out in a whole cell environment wherein the cell is transformed to express both the oxygenase being screened and the coupling enzyme. The method can be used to measure the activity of monooxygenases and dioxygenases on aromatic substrates. The method is amenable to large scale screening of enzyme mutants to isolate those with maximum oxygenase activity.

WO 02/22861 A1

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

OXYGENASE ENZYMES AND SCREENING METHOD

The Government has certain rights to this invention pursuant to Grant No. N0014-96-1-0340, awarded by the United States Navy.

This application is a continuation-in-part of U.S. application No. 09/246,451, which claims priority from U.S. application No. 60/094,403, filed on July 28, 1998; No. 60/106,840 filed on November 3, 1998; No. 60/086,206 filed May 21, 1998; and No. 60/106,834 filed on November 3, 1998.

BACKGROUND OF THE INVENTION

Field of the Invention

The publications and reference materials noted herein and listed in the appended Bibliography are each incorporated by reference in their entirety.

The invention relates to enzymes, called oxygenases, which are biologically active proteins that catalyze certain oxidation reactions involving the addition of oxygen to a substance. The transfer of oxygen from an oxygen-donor compound, such as molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), to any of millions of useful aromatic or aliphatic substrate compounds is important in organic chemistry and in many biochemical reactions. Typical oxidation reactions include hydroxylation, epoxidation and sulfoxidation, which are widely used in the production of chemicals including pharmaceuticals and other compounds used in medicine. Enzymes which catalyze or improve oxidation reactions are useful in science and industry. The invention relates to novel oxygenase enzymes having improved properties. The invention also relates to methods of screening for oxygenase enzymes, and more particularly, to methods for identifying oxidation enzymes which exhibit catalytic activity with respect to the insertion of oxygen into aromatic or aliphatic compounds.

The screening method involves introducing an organic substrate compound to an oxygen donor compound in the presence of a test enzyme. Exemplary oxygen donors include molecular oxygen or dioxygen (O_2) and peroxides such as hydrogen peroxide (H_2O_2) and t-butyl peroxide. Exemplary substrates include naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, and anthracene. An oxygenated product is formed when the test enzyme has oxidation activity, particularly oxygenase activity, under test conditions.

A coupling enzyme is used to bring together molecules of the oxygenated product into larger molecules or polymers which absorb UV light, produce a color change, or are fluorescent or luminescent. Exemplary coupling enzymes include peroxidases from various microbial and plant sources, such as horseradish peroxidase (HRP), cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, peroxidase Novozyme® 502, as well as laccases such as fungal laccase. The presence and degree of a change in absorbance, color, fluorescence or luminescence can be detected or measured, and indicates the presence of oxygenated product. Detection can be enhanced by a chemiluminescent agent, such as luminol. These techniques provide a reliable indication of oxygenase activity, that is, the production of oxygenated compound by reaction of the oxygen donor with the substrate in the presence of (and mediated by) the enzyme.

The method is preferably carried out in a whole cell environment. A host cell is transformed, using genetic engineering techniques, to express an oxygenase being screened, and may also be engineered to express a coupling enzyme. The method is amenable to large scale screening of enzyme mutants to isolate those with desirable oxygenase activity, for example maximum activity under certain conditions or towards a particular substrate compound. The method is also amenable to screening gene libraries isolated from nature (50).

Oxygenase enzymes typically use molecular oxygen, in the presence of cofactors, coenzymes, and/or ancillary proteins, to add oxygen to a substrate. Oxygen is a highly reactive chemical element. In pure molecular form, it is a gas that is a principal component of air, and is stable as a combination of two oxygen atoms (O_2). It appears in water (H_2O), in rocks and minerals, in many organic compounds, and is active in many biochemical and physiological processes. Some O_2-utilizing enzymes can use other oxygen donors, *e.g.* peroxides (according to a reaction scheme called the peroxide shunt pathway), but do so poorly, with low activity and a low yield of oxygenated product. Moreover, certain coenzymes, cofactors or ancillary proteins may still be required, although the peroxide shunt does not require the difficult coenzymes, *e.g.* NAD(P)H, associated with pathways using O_2 as a substrate.

The improved oxygenase enzymes of the invention are capable of efficiently catalyzing reactions wherein oxygen is added to a substrate, using oxygen donors other than molecular oxygen, and without requiring certain cofactors, coenzymes, or ancillary redox

proteins. These new enzymes have significantly more activity than native enzymes. For example, they are at least twice as active, and typically are ten or more times as active as a wild-type enzyme towards a particular substrate or under particular reaction conditions.

Description of Related Art

The publications and reference materials noted here and in the appended Bibliography are each incorporated by reference in their entirety. They are referenced numerically in the text and the Bibliography below.

Catalysts, Enzymes and Oxygenases. An enzyme is a biological catalyst, typically a protein, which promotes a biochemical reaction. A catalyst enables a chemical reaction to proceed at a faster rate or under different conditions than would otherwise occur. Usually, a catalyst is itself unchanged at the end of the reaction, although oxidative enzymes may be deactivated slowly during these reactions. Oxygenase enzymes that are capable of catalyzing the insertion of oxygen into aromatic (ring-containing) and aliphatic (open-chain) chemical compounds, and other chemical compounds or substrates have many potential applications in pharmaceuticals manufacturing, in the production of chemicals, and also in medicine. Dioxygenases introduce two atoms of oxygen, *e.g.* both oxygens from a donor such as molecular oxygen (O_2). Monooxygenases, also called mixed function oxygenases, add one atom of oxygen to a substrate compound. In these reactions a second oxygen from the oxygen donor may be combined with hydrogen (H^+) in a companion reaction, called a reduction reaction, to form water (H_2O). Compounds other than molecular oxygen, such as peroxides, can also donate oxygen to a substrate in the presence of various oxygenases.

Common monooxygenation reactions include hydroxylation and epoxidation. In a hydroxylation reaction, oxygen is introduced to a substrate as a hydroxyl group (OH). In an epoxidation reaction, oxygen is introduced as a bridge across two other atoms, typically in place of a double bond between two carbon atoms. This can form an activated or reactive group having a three-member ring of one oxygen atom and two carbon atoms. A common dioxygenation reaction is sulfoxidation. In a sulfoxidation reaction, two oxygen atoms are added to a sulfur atom that is bonded to two other atoms, typically two carbon atoms, each of which is part of a hydrocarbon chain.

The introduction of oxygen to a compound may change its biochemical activity or functionality, and may activate the compound so that it can participate in further chemical reactions. Oxygenated substrates may be used by organisms or industrially, in the synthesis

of useful compounds from starting materials or intermediates. Oxygenation may also be useful in the breakdown of compounds, to provide starting materials and intermediates for other reactions. For example, bacteria use oxygenases to digest aromatic compounds.

Problems Addressed by the Invention. Among the problems addressed by the invention are the significant disadvantages of many known enzyme systems. These problems have prevented commercial use and exploitation of such systems. Many oxygenases, like other enzymes, require expensive coenzymes (*e.g.* NADPH) and ancillary proteins (*e.g.* a reductase enzyme), and often must be used in whole cells or reactors with recycled coenzymes, to keep the coenzyme costs low. Known enzymes also are relatively inefficient or unstable under industrial conditions, and may be undesirably deactivated by reaction products or byproducts, or for other reasons. These types of enzyme systems, particularly when used in whole cell reactions, are also prone to competing reactions which can lower the selectivity and yield.

Thus, enzymes which do not require coenzymes, use less coenzymes, or use less expensive coenzymes are desirable. Enzymes which are more efficient, more stable, or which function under different conditions are also desirable. It would also be desirable to provide enzymes which are not adversely affected by competing reactions. Enzymes which promote oxidation of different substrates, which insert oxygen at different positions on a given substrate, insert oxygen more efficiently, or use different oxygen donor compounds would also be desirable, as would enzymes which are more or less specific than known enzymes in catalyzing certain reactions. For example, hydrogen peroxide or other peroxides are good choices of oxidant for fine chemicals manufacturing, as their use would require less specialized equipment, and less cost overall, than molecular oxygen due to the greatly simplified catalyst system. A suitable screening method for oxygenases is also desirable, and would provide an important tool in the discovery and identification of new and improved oxidation enzymes.

Enzymatic oxygenation reactions are particularly intriguing, because directed oxyfunctionalization of unactivated organic substrates remains a largely unresolved challenge to synthetic chemistry. This is especially true for regiospecific reactions, where oxygenation at a specific position of a substrate occurs in only one of two or more possible ways. For example, regiospecific hydroxylation of aromatic compounds by purely chemical methods is notoriously difficult. Reagents for ortho or *o*-hydroxylation of ring compounds, at positions on the ring which are next or adjacent to each other, are described in the

literature. Reagents are also available for para or *p*-hydroxylation, at positions on the ring which are opposite each other. However, some of these reagents are explosive, and undesirable by-products are usually obtained (1). Likewise, specific oxygenation of enantiomers (mirror-image forms of a compound), is difficult and not well understood. In these reactions, one enantiomer is preferentially oxygenated, but the mirror-image enantiomer of the same compound is poorly oxygenated, or is not oxygenated at all. Similarly, it is difficult to oxygenate a substrate with high enantiospecificty, *i.e.* so as to create one particular enantiomeric form versus another. Thus, oxygenation to form a particular enantiomer is difficult. Consequently, oxidation enzymes which facilitate particular regiospecific or enantiospecific reactions would be desirable, particularly enzymes which do so under laboratory or industrial conditions, or which do so more efficiently or in some better way.

Oxidation Enzymes. Various native mono- and dioxygenase enzymes from different microbial, human, plant, and animal sources are known. These include enzymes such as chloroperoxidase (CPO), large numbers of cytochrome P450 enzymes (P450), methane monooxygenases (MMO), toluene monooxygenases, toluene dioxygenases (TDO), biphenyl dioxygenases and naphthalene dioxygenases (NDO). These enzymes have demonstrated the ability to catalyze hydroxylation and many other interesting and useful oxidation reactions. However, they are generally unsuitable for industry due to their inherent complexity, low stability and low productivity under industrial conditions (*e.g.* in the presence of organic solvents, high concentrations of reactants, etc.).

One class of known oxidation enzymes is the cytochrome P450 enzymes. These heme proteins have iron-containing heme groups and are important monooxygenase enzymes involved in, among other reactions, detoxification of foreign or toxic materials (xenobiotics), drug metabolism, carcinogenesis, and steroid biosynthesis (5 and 6). One exemplary P450 enzyme, $P450_{cam}$ from *Pseudomonas putida*, whose natural substrate is camphor, is also capable of regiospecific hydroxylation of a variety of substrates including, at a low level of activity, naphthalene ($C_{10}H_8$), a bicyclic aromatic compound (7). However, the catalytic turnover of this enzyme requires the reduced form of nicotinamide-adenine dinucleotide (NADH) as a coenzyme and two ancillary proteins. One of these proteins is putidāredoxin, an iron-sulfur protein (also called a ferredoxin) that acts as an electron carrier to shuttle electrons from NADH. The other ancillary protein is the enzyme putidaredoxin reductase, a flavoprotein which catalyzes the transfer of hydrogen atoms from one substrate to another

(8). This requirement for two redox proteins and NADH makes $P450_{cam}$ and other P450 catalysis highly expensive and difficult to use in laboratory and industrial applications. It would be desirable to provide a simpler and more economical P450-type catalyst and hydroxylation system, in particular a system which requires fewer ancillary proteins or coenzymes, or which does not require them at all.

P450 enzymes typically use dioxygen (O_2) as the oxygen donor for hydroxylation, adding one oxygen to a substrate compound, such as naphthalene, and forming water with hydrogen and another oxygen as a byproduct. They are most efficient when using dioxygen with expensive coenzymes, such as the reduced forms of nicotinamide-adenine dinucleotide (NADH) or nicotinamide-adenine dinucleotide phosphate (NADPH), collectively "NAD(P)H". Ancillary proteins may also be needed for efficient enzyme activity. However, various P450s (and, possibly, some MMOs) are able to catalyze the hydroxylation of an organic substrate using a peroxide, such as hydrogen peroxide or alkyl peroxides, via the so-called peroxide shunt pathway (9). Peroxides are compounds, other than molecular O_2, in which oxygen atoms are joined to each other. Other oxygen donors include peroxyacids, $NaIO_4$, $NaClO_2$, and iodosyl benzene.

Nordblom et al. (11) studied hydroperoxide-dependent substrate hydroxylation by liver microsomal P450 in hepatic microsomes. A variety of substrates were shown to be attacked by the enzyme in the presence of cumene hydroperoxide. Using benzphetamine as the substrate, it was also shown that other peroxides, including hydrogen peroxide, peracids and sodium chlorite, could be used in place of oxygen (11). *Rahimtula et al.* (12) showed that cumene hydroperoxide is capable of supporting the hydroxylation of various aromatic compounds (biphenyl, benzpyrene, coumarin, aniline) by cytochrome P450 in hepatic microsomes. Unfortunately, native cytochrome P450 is rapidly deactivated by peroxides and other oxidants. The enzyme chloroperoxidase (CPO) from *Caldariomyces fumago* has an active site whose structure is similar to cytochrome P450 enzymes. CPO will catalyze various oxidation reactions, including enantioselective hydroxylation, epoxidation and sulfoxidation, using peroxides. This enzyme utilizes peroxide efficiently but cannot utilize molecular oxygen because it does not have the coenzyme machinery of the P450 enzymes. CPO also provides an example of an enzyme that is deactivated by reactive intermediates. Heme alkylation by the epoxide product in the CPO-catalyzed epoxidation of 1-alkenes results in CPO deactivation.

Heme oxygenases such as P450s and heme peroxidases, which are peroxidase enzymes that contain the heme prosthetic group, are generally prone to deactivation via oxidation of the porphyrin ring in the heme substrate, by reaction with so-called suicide inhibitors formed during catalysis, and also by formation of Compound III (for peroxidases). Compound III is an intermediate enzyme-substrate-oxygen-iron complex, sometimes referred to an oxyperoxidase. For example, the enzyme horseradish peroxidase (HRP) is deactivated during the oxidation of phenol compounds, *e.g.* six-member hydrocarbon ring structures containing one or more hydroxyl (OH) groups. In theory, this may be due to the formation of phenoxy radicals which react with oxygen to form a reactive peroxy-radical species. Compound III forms in the presence of excess hydrogen peroxide and is not involved in the reaction cycle. However, its accumulation reduces the amount of active enzyme. Compound III stability in turn depends on the specific enzyme.

The rates of all of these deactivation pathways depend on the protein framework, *i.e.* the particular proteins, structures and conditions involved. They all are therefore amenable to improvement by mutations. This includes oxygenases that are more suitable to function in the presence of high concentrations of hydrogen peroxide, or other peroxides or oxygen-donating agents. Improved oxygenases also include those which are more resistant to deactivation, do not require coenzymes or use them more efficiently, function under different conditions or with different specificities, or which hydroxylate different substrates or a variety of substrates, or which do so more efficiently. As one example, it would be desirable to make modified P450 enzymes that are functionally similar or equivalent to CPO, or which share desirable features of CPO. An improved P450 enzyme of this kind, for example, would have the ability to oxygenate a substrate or substrates using a peroxide, *e.g.* hydrogen peroxide, without expensive coenzymes, and with a high efficiency and improved resistance to deactivation.

Enzyme Modification. The observed constraints on the use of native enzymes are thought to be a consequence of evolution. Enzymes have evolved in the context and environment of a living organism, to carry out specific biological functions under conditions conducive to life – not laboratory or industrial conditions. In some cases, evolution may favor or even require less than optimally efficient enzymes. For example, detoxication enzymes, such as cytochrome P450 enzymes, function to help convert foreign (xenobiotic) chemical compounds into other compounds that an organism can use, that are not toxic, or that are present in non-toxic amounts. In order to deal with environmental conditions or

foreign compounds an organism has not encountered before, detoxification enzymes may attack a relatively large number of substrates, and may accidentally produce products that are as or more toxic than the substrate. Thus, maximizing the flow of potentially harmful foreign substrates for processing, *e.g.* using an overly efficient catalyst, may not be the best evolutionary strategy. This is particularly true when there is a time-dependent xenobiotic profile, meaning that the organism can only safely handle so much foreign material at a time (2). In this situation, a less than maximally active enzyme that is appropriately balanced to the particular needs of the organism and its environment would be a better evolutionary goal. In a laboratory or industrial setting, it is desirable to provide enzymes which are more active, and process more substrate more rapidly.

Thus, the output, efficiency, working conditions, stability and other properties of known enzymes are not thought to be unalterable, nor are they limitations which are seen as intrinsic to the nature of these catalysts as proteins. It is possible that these native catalysts can be evolved *in vitro*, or that analogous catalysts can be otherwise developed, to alter or enhance the enzyme's properties, for example to obtain much more efficient laboratory or industrial oxidative catalysts. Enzyme selectivity and substrate specificity may also be altered to better match the needs of the synthetic chemist. Improved catalysts can also be obtained by screening cultures of native organisms or expressed gene libraries (3).

One technique which may be applied to the discovery of improved catalytic enzymes is directed evolution. Directed evolution is a procedure by which the evolutionary process is accelerated *in vitro* to produce mutant enzymes which have certain desired characteristics. An example of the use of directed evolution for identifying and isolating improved para-nitrobenzyl esterases is set forth in U.S. Patent No. 5,741,691. *See also,* U.S. Patent No. 5,811,238 (13). Other techniques, such as random mutagenesis, may also be used to obtain new enzymes. Improved enzymes may also be discovered in nature.

According to a preferred embodiment of the invention, directed evolution or random mutagenesis can be used to produce an array of efficient catalysts which can perform oxidations using agents other than dioxygen (O_2) as the oxidant. For example, peroxides such as hydrogen peroxide (H_2O_2) may be used. Directed evolution can also be used to alter the properties of oxidative enzymes that use molecular oxygen. A variety of such enzymes, including cytochrome P450s, other monooxygenases, and dioxygenases such as toluene dioxygenase, facilitate useful oxygenation reactions. It is desirable to alter the reactivities, selectivities and stabilities of these enzymes to produce improved enzymes. An important

tool for finding improved oxidation biocatalysts in nature, by directed evolution, by random mutagenesis, or by other means, is a sensitive, accurate and rapid screening method. Accordingly, there is a need to develop new and improved screening methods for enzymes which function as oxygenases. In particular there is a need for screening methods which are well-suited for use in connection with directed evolution procedures.

SUMMARY OF THE INVENTION

In accordance with the invention, a method of screening for oxidation enzymes or oxygenases is provided. New and improved oxidation enzymes are also provided.

More particularly, the presence of oxygenated compounds which are produced by the action of an oxygenase on a particular substrate is detected. The invention is particularly well suited for screening large numbers of both naturally occurring and mutated oxygenases to determine their activity with respect to a wide range of substrates, including aromatic and aliphatic compounds. It was discovered that the detection of oxygenated compounds produced by action of an oxygenase can be improved by reacting the oxygenated compound with a coupling enzyme to form a polymeric oxygenated compound which absorbs UV light, produces a color change, or is luminescent, *i.e.* phosphorescent or preferably fluorescent. The presence and amount of oxygenated compounds in a sample can be indicated by detecting, observing or measuring the presence, and if desired the degree, of light absorption, color change, fluorescence, or luminescence. It was also discovered that the luminescence and detection of the polymeric oxygenated compound can be further enhanced by creating the polymeric oxygenated compound in the presence of a chemiluminescent agent, such as luminol, to increase chemiluminesence intensity and/or lifetime. Other agents can also be used to enhance color development or color change reactions (44).

The invention is particularly well suited for whole cell screening procedures wherein a host cell, such as the *E. coli* bacteria, is transformed with a suitable vector to express an oxygenase to be screened. The transformed cell is treated with a substrate, such as naphthalene, for a sufficient time to allow an oxygenated compound, e.g., hydroxylated naphthalene, to be formed. A coupling enzyme, such as horseradish peroxidase (HRP), is provided and allowed to react with the oxygenated compound, to form a polymeric oxygenated compound which exhibits increased levels of UV light absorption, luminescence, or fluorescence in the case of polymeric hydroxylated naphthalene. The fluorescence generated by the polymeric oxygenated compound is measured by known means to provide

indirect detection of the activity of the oxygenase, *e.g.*, the amount of oxygenated compound produced by reaction of the oxygenase with the substrate. The coupling enzyme can be produced extraneously and added to the cell culture, or in a preferred embodiment, it can be produced intracellularly, that is, by or inside the same cell that is producing the oxygenase.

Thus, a whole cell screening system is provided wherein a suitable host cell is transformed with suitable vectors to provide co-expression by the transformed cell of both an oxygenase and a coupling enzyme. As a result, infusion of the substrate into the cell results in contemporaneous generation of oxygenated compounds due to action of the oxygenase on the substrate and the formation of polymeric oxygenated compounds resulting from action of the coupling enzyme on the oxygenated compounds. When desired, one or more cofactors, coenzymes or ancillary proteins can be used to improve the activity of the oxidation enzyme or enhance the oxygenation reaction.

The invention is particularly well suited for screening a large number of naturally occurring or mutated oxygenases to determine relative enzyme activities with respect to a substrate, and in particular to establish which enzymes exhibit the highest activity with respect to a given substrate or which insert oxygen at a different site on the substrate (show different regiospecificity). The invention is applicable to both monooxygenases or dioxygenases and can be used to detect oxygenated compounds formed by hydroxylation or epoxidation. The invention can also be applied to sulfoxidation and hydroxylation reactions. Hydroxylation enzymes are one preferred species of enzymes for the invention.

The invention is also suitable for screening libraries of oxygenase catalysts that are not enzymes, for example, compounds generated by combinatorial chemistry (43, 48, 49). The addition of oxygen by such catalysts can be assayed by addition of a coupling enzyme under conditions suitable for the coupling reaction. For example, conditions can be modified after the oxygenation reaction to accommodate the coupling reaction. However, it may not be necessary to significantly modify the reaction conditions for some coupling enzymes. As one example, horseradish peroxidase is known to function over a wide range of conditions and in aqueous media and in a wide variety of nonpolar organic solvents.

The above features and many other attendant advantages of the invention will become better understood by reference to the following detailed description when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of a reaction pathway of an exemplary embodiment of the invention.

FIG. 2 is a map of an exemplary vector used to express the wild type cytochrome $P450_{cam}$ oxygenase and its mutants in *E. coli*.

FIG. 3A shows a nucleotide coding sequence for wild type cytochrome $P450_{cam}$ monooxygenase **[SEQ ID NO: 1]**. **FIG. 3B** shows an amino acid sequence for wild type cytochrome $P450_{cam}$ monooxygenase **[SEQ ID NO: 2]**.

FIG. 4A is a pictorial representation of an exemplary 96 well plate assay in accordance with the invention. **FIG. 4B** is a diagrammatic representation of a reaction scheme according to the invention.

FIG. 5A is a tabular representation of the wells in a 96-well plate in which different media and components were used to evaluate the effect on the $P450_{cam}$ activity of transformed *E. coli* host cells. **FIG. 5B** is a graphic representation of $P450_{cam}$ activity as measured by an assay according to the invention. Each column of the graph represents the total $P450_{cam}$ activity in each corresponding well of the 96 well plate, as a measure of the fluorescence produced by the polymerized oxygenated reaction products of naphthalene hydroxylated by hydrogen peroxide in the presence of the $P450_{cam}$ and HRP enzymes.

FIG. 6 is a pictorial representation of an exemplary assay according to the invention.

FIG. 7 is a pictorial representation of how simultaneous expression of the oxygenase and coupling enzyme in *E. coli* leads to generation of fluorescent cells.

FIG. 8 shows development of fluorescence, over time, in whole cells transformed to co-express P450 oxygenase and HRP coupling enzyme with naphthalene substrate and hydrogen peroxide oxygen donor (●); without naphthalene substrate (❑); and without oxygen donor (◴). For comparison, fluorescence was also evaluated in whole cells transformed to express HRP without P450 (□), P450 without HRP (■) and host cell that were not transformed (◴).

FIG. 9 shows the effect of inducer levels on co-expression and production of P450 enzyme and HRP enzyme in *E. coli* host cells, according to a preferred embodiment of the invention.

FIG. 10 shows the fluorescence of colonies of induced *E. coli* host cells transformed to co-express P450 and HRP enzymes, in the presence of naphthalene and hydrogen peroxide.

FIGS. 11A-11D show the computer-assisted image analysis of a group of colonies of fluorescent cells in a whole cell P450/HRP assay according to the invention.

FIGS. 12A-12F show the image analysis results, in graphic form, of the fluorescence shown by colonies of *E. coli* host cells (control), and the same host cells transformed to express P450 enzyme, HRP enzyme, or both, and under different assay conditions (with and without substrate and oxygen donor).

FIG. 13 depicts a method for automatically detecting positive (fluorescent) colonies of whole cells which produce active oxygenase enzyme according to the invention. Colonies of host cells are plated, and the plate is conceptually divided by a grid into rectilinear compartments, each of which can be scanned by conventional image analysis equipment (**FIG. 13A**). Each compartment is scanned for fluorescent colonies (**FIG. 13B**) and the number of positive (fluorescent) colonies counted. The fluorescence intensity is also measured (**FIG. 13C**). Colonies containing improved oxygenases (fluorescence above a certain level) can be identified and selected. This technique can be automated.

FIG. 14A shows the results of a experiment using coumarin as a substrate for oxygenation, in an assay of the invention. **FIG. 14B** shows the results of an experiment using 3-phenyl propionate as the substrate.

FIG. 15 shows a 96 well plate assay according to the invention, in which the fluorescence of 3-phenyl propionate substrate oxygenated and polymerized in an *E. coli* P450/HRP co-expression whole cell system is amplified using luminol. A comparison is shown with a host cell control, and with cells transformed to express P450 enzyme without HRP and HRP enzyme without P450. Results using ultraviolet (UV) irradiation are shown in **FIG. 15A**. Results without irradiation are shown in **FIG. 15B**.

FIG. 16 shows the yeast cytochrome c peroxidase (CCP) expression vector pet-26b(+)CCP.

FIG. 17 shows the detection of fluorescence in an embodiment of the invention in which cytochrome c peroxidase (CCP) is used as a coupling enzyme that is co-expressed with P450 enzyme in a whole cell system. Comparisons with an *E. coli* host cell control, without substrate, and with cells transformed to express CCP without P450 and P450 without CCP are also shown.

FIG. 18 shows the toluene dioxygenase (TDO) expression vector pXTD14.

FIG. 19A shows the results of a digital scan of a section of a plate containing fluorescent mutant $P450_{cam}$ colonies. **FIG. 19B** shows the results of about 32,000 clones

from a digital scan of about 200,000 clones from plates containing mutant $P450_{cam}$ colonies. FIG. 19C-19F shows a graphical representation of the P450 enzyme activities of a sample of mutant $P450_{cam}$ colonies as measured by fluorescence, in an assay of the invention.

FIG. 20 shows the results of measuring the fluorescence of 96 randomly selected clones from the large mutant library (about 20,000 colonies) in a screen according to the invention.

FIG. 21 is a map of an exemplary vector, pETpelBHRP, formed by inserting the HRP gene into the plasmid pET-22b(+), which contains a T7 promoter and a pelB signal sequence. The resulting vector was used as the starting point for mutagenesis to express horseradish peroxidase in *E. coli* host cells.

FIG. 22 shows the coding sequence of the pelB signal peptide ([SEQ ID NO: 14]) and ([SEQ. ID. NO. 15].

FIG. 23 shows a nucleotide and amino acid sequence encoding a recombinant wild-type HRP enzyme designated HRP1A6 ([SEQ. ID NO. 16 and SEQ. ID. NO. 17]).

FIG. 24 is a map of the expression vector pETpelBHRP1A6.

FIG. 25 is a map of the expression vector pYEXS1-HRP containing a coding sequence for HRP cloned into the secretion plasmid pYEX-S1.

DETAILED DESCRIPTION OF THE INVENTION

The invention concerns oxidation enzymes and a general method for screening enzymes that are capable of oxygenating various substrates. In particular, the invention is especially well suited for evaluating the activity of enzymes that are capable of oxygenating aromatic substrates.

Definitions

As used herein, "about" or "approximately" shall mean within 50 percent, preferably within 20 percent, more preferably within 5 percent, and even more preferably within 5 percent of a given value or range.

The term "polymer" means any substance or compound that is composed of two or more building blocks ('mers') that are repetitively linked to each other. For example, a "dimer" is a compound in which two building blocks have been joined together.

A "protein" or "polypeptide", which terms are used interchangeably herein, comprises one or more chains of chemical building blocks called amino acids that are linked together by chemical bonds called peptide bonds.

An "enzyme" means any substance, preferably composed wholly or largely of protein, that catalyzes or promotes, more or less specifically, one or more chemical or biochemical reactions. The term "enzyme" can also refer to a catalytic polynucleotide (e.g. RNA or DNA). A "test" enzyme is a substance that is tested to determine whether it has properties of an enzyme.

Proteins and enzymes can be made in a host cell using instructions in DNA and RNA, according to the genetic code. "Transcription" is the process by which a DNA sequence or gene having instructions for a particular protein or enzyme is "transcribed" into a corresponding sequence of RNA. "Translation" is the process by which the RNA sequence is "translated" into the sequence of amino acids which form the protein or enzyme.

A "native" or "wild-type" protein, enzyme, polynucleotide, gene, or cell, means a protein, enzyme, polynucleotide, gene, or cell that occurs in nature.

A "parent" protein, enzyme, polynucleotide, gene, or cell, is any protein, enzyme, polynucleotide, gene, or cell, from which any other protein, enzyme, polynucleotide, gene, or cell, is derived or made, using any methods, tools or techniques, and whether or not the parent is itself native or mutant. A parent polynucleotide or gene can encode for a parent protein or enzyme.

A "mutant", "variant" or "modified" protein, enzyme, polynucleotide, gene, or cell, means a protein, enzyme, polynucleotide, gene, or cell, that has been altered or derived, or is in some way different or changed, from a parent protein, enzyme, polynucleotide, gene, or cell. A mutant protein or enzyme is usually, although not necessarily, expressed from a mutant polynucleotide or gene.

A "mutation" means any process or mechanism resulting in a mutant protein, enzyme, polynucleotide, gene, or cell. This includes any mutation in which a protein, enzyme, polynucleotide, or gene sequence is altered, any protein, enzyme, polynucleotide, or gene sequence arising from a mutation, any expression product (*e.g.* protein or enzyme) expressed from a mutated polynucleotide gene sequence, and any detectable change in a cell arising from such a mutation.

Regarding genetic material, "mutant" and "mutation" includes polynucleotide alterations arising within a protein-encoding region of a gene as well as alterations in regions outside of a protein-encoding sequence, such as, but not limited to, regulatory sequences. "Mutant" also includes a "silent" mutant and "sequence-conservative variants", which is a mutant polynucleotide sequence that, upon translation, is not reflected in an altered amino

acid sequence. Such silent mutations can occur when one amino acid corresponds to more than one codon.

"Function-conservative variants" are proteins or enzymes in which a given amino acid residue has been changed without altering overall conformation and function of the protein or enzyme, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, acidic, basic, hydrophobic, and the like). Amino acids with similar properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Amino acids other than those indicated as conserved may differ in a protein or enzyme so that the percent protein or amino acid sequence similarity between any two proteins of similar function may vary and may be, for example, from 70% to 99% as determined according to an alignment scheme such as by the Cluster Method, wherein similarity is based on the MEGALIGN algorithm. A "function-conservative variant" also includes a polypeptide or enzyme which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75%, most preferably at least 85%, and even more preferably at least 90%, and which has the same or substantially similar properties or functions as the native or parent protein or enzyme to which it is compared.

A "functional" protein or enzyme is capable of displaying biological activity, such as, for example, participating in a designated biochemical reaction. Generally, a screening test can be used to detect and/or evaluate whether a protein is functional or not.

A "property" of a protein or enzyme, wild-type or mutated, means a feature, preferably detectable in a screening test, associated with the protein. Protein properties include, but are not limited to, the ability of the protein to fold correctly, the stability of the protein in a certain media and/or over time, the expression level or yield of a protein expressed by a host cell, functionality (*i.e.*, whether the protein is functional or non-functional), and, in the case of a enzyme, enzyme activity.

The "activity" of an enzyme is a measure of its ability to catalyze a reaction, and may be expressed as the rate at which the product of the reaction is produced. For example, enzyme activity can be represented as the amount of product produced per unit of time, per unit (*e.g.* concentration or weight) of enzyme.

The "stability" of an enzyme means its ability to function, over time, in a particular environment or under particular conditions. One way to evaluate stability is to assess its

ability to resist a loss of activity over time, under given conditions. Enzyme stability can also be evaluated in other ways, for example, by determining the relative degree to which the enzyme is in a folded or unfolded state. Thus, one enzyme is more stable than another, or has improved stability, when it is more resistant than the other enzyme to a loss of activity under the same conditions, is more resistant to unfolding, or is more durable by any suitable measure. For example, a more "thermally stable" or "thermostable" enzyme is one that is more resistant to loss of structure (unfolding) or function (enzyme activity) when exposed to heat or an elevated temperature. One way to evaluate this is to determine the "melting temperature" or T_m for the protein. The melting temperature, also called a midpoint, is the temperature at which half of the protein is unfolded from its fully folded state. This midpoint is typically determined by calculating the midpoint of a titration curve that plots protein unfolding as a function of temperature. Thus, a protein with a higher T_m requires more heat to cause unfolding and is more stable or more thermostable. Stated another way, a protein with a higher T_m indicates that fewer molecules of that protein are unfolded at the same temperature as a protein with a lower T_m, again meaning that the protein which is more resistant to unfolding is more stable (it has less unfolding at the same temperature). Another measure of stability is $T_{1/2}$, which is the transition midpoint of the inactivation curve of the protein as a function of temperature. $T_{1/2}$ is the temperature at which the protein loses half of its activity. Thus, a protein with a higher $T_{1/2}$ requires more heat to deactivate it, and is more stable or more thermostable. Stated another way, a protein with a higher $T_{1/2}$ indicates that fewer molecules of that protein are inactive at the same temperature as a protein with a lower $T_{1/2}$, again meaning that the protein which is more resistant to deactivation is more stable (it has more activity at the same temperature). These assays are also called "thermal shift" assays, because the inactivation or unfolding curve, plotted against temperature, is "shifted" to higher or lower temperatures when stability increases or decreases. Thermostability can also be measured in other ways. For example, a longer half-life ($t_{1/2}$) for the enzyme's activity at elevated temperature is an indication of thermostability.

The term "substrate" means any substance or compound that is converted or meant to be converted into another compound by the action of an enzyme catalyst. The term includes aromatic and aliphatic compounds, and includes not only a single compound, but also combinations of compounds, such as solutions, mixtures and other materials which contain at least one substrate. Aromatic substrates are preferred. Exemplary and non-limiting aromatic substrates of the invention include naphthalene, 3-phenylpropionate (3-

PPA), coumarin, benzene, toluene, and benzoic acid. Preferred substrates, particularly in connection with the screening methods of the invention, are naphthalene and 3-phenylpropionate.

The term "cofactor" means any non-protein substance that is necessary or beneficial to the activity of an enzyme. A "coenzyme" means a cofactor that interacts directly with and serves to promote a reaction catalyzed by an enzyme. Many coenzymes serve as carriers. For example, NAD^+ and $NADP^+$ carry hydrogen atoms from one enzyme to another. An "ancillary protein" means any protein substance that is necessary or beneficial to the activity of an enzyme.

An "oxidation reaction" or "oxygenation reaction", as used herein, is a chemical or biochemical reaction involving the addition of oxygen to a substrate, to form an oxygenated or oxidized substrate or product. An oxidation reaction is typically accompanied by a reduction reaction (hence the term "redox" reaction, for oxidation and reduction). A compound is "oxidized" when it receives oxygen or loses electrons. A compound is "reduced" when it loses oxygen or gains electrons. According to the invention, oxidation reactions are preferably oxygenation reactions which add oxygen to a substrate. Oxygen typically donates electrons in ionic form as OH^- or O_2^{2-}. Conceptually, electrons (negatively charged subatomic particles) may also be lost or gained via the transfer of protons (positively charged subatomic particles), for example as hydrogen ions (H^+ of H_2^{2+}). An "ion" is an atom or molecule with a net positive or negative charge, *i.e.* it has excess electrons (a negative charge) or is missing electrons (a positive charge). Thus, an oxidation reaction can also be called an "electron transfer reaction" and encompass the loss or gain of electrons (*e.g.* oxygen) or protons (*e.g.* hydrogen) from a substance. Preferred oxidized compounds of the invention are those which are "oxygenated", meaning they have received oxygen.

The terms "oxygen donor", "oxidizing agent" and "oxidant" mean a substance, molecule or compound which donates oxygen to a substrate in an oxidation reaction. Typically, the oxygen donor is reduced (accepts electrons). Exemplary oxygen donors, which are not limiting, include molecular oxygen or dioxygen (O_2) and peroxides, including alkyl peroxides such as t-butyl peroxide, and most preferably hydrogen peroxide (H_2O_2). A peroxide is any compound having two oxygen atoms bound to each other.

An "oxidation enzyme" is an enzyme that catalyzes one or more oxidation reactions, typically by adding, inserting, contributing or transferring oxygen from a source or donor to a substrate. Such enzymes are also called oxidoreductases or redox enzymes, and

encompasses oxygenases, hydrogenases or reductases, oxidases and peroxidases. An "oxygenase" is an oxidation enzyme that catalyzes the addition of oxygen to a substrate compound. A "dioxygenase", is an oxygenase enzyme that adds two atoms of oxygen to a substrate. A "monooxygenase" adds one atom of oxygen to a substrate. An "oxidase" is an oxidation enzyme that catalyzes a reaction in which molecular oxygen (dioxygen or O_2) is reduced, for example by donating electrons to (or receiving protons from) hydrogen.

Preferred oxidation enzymes of the invention include, without limitation, oxygenases (dioxygenases and monooxygenases), including hydroxylases, epoxidases, and sulfoxidases, which catalyze, respectively, hydroxylation, epoxidation, and sulfoxidation reactions. Of these, monooxygenases, hydroxylases, and dioxygenases are preferred. Exemplary oxidation enzymes include, without limitation, native or modified chloroperoxidase (CPO), cytochrome P450s, methane monooxygenases (MMOs), toluene monooxygenase, toluene dioxygenases (TDO), naphthalene dioxygenases (NDO), and biphenyl dioxygenases. A preferred oxidation enzyme is native or modified cytochrome P450.

The term "coupling enzyme" means an enzyme which catalyzes a chemical or biochemical reaction in which an oxygenated substrate or product reacts to forms a detectable complex, aggregate, polymer, other reaction product. A preferred coupling enzyme catalyzes the formation of a reaction product that has a detectable or enhanced color change, UV absorbance or luminescence (*e.g.* fluorescence). For example, a suitable coupling enzyme catalyzes the formation of a fluorescent polymer by joining two or more oxygenated substrate molecules to each other. According to one embodiment of the invention, the fluorescence of the polymerized oxygenated compound is more readily detectable than the fluorescence, if any, of oxygenated substrate which has not been polymerized. A coupling enzyme may or may not be an oxidation enzyme, provided it functions to catalyze the formation of a detectable oxygenated reaction product. Exemplary coupling enzymes include, without limitation, peroxidases from various microbial and plant sources, such as horseradish peroxidase (HRP), cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, peroxidase Novozyme® 502, as well as laccases such as fungal laccase. HRP and laccase are preferred coupling enzymes.

A "luminescent" substance means any substance which produces detectable electromagnetic radiation, or a change in electromagnetic radiation, most notably visible light, by any mechanism, including color change, UV absorbance, fluorescence and

phosphorescence. Preferably, a luminescent substance according to the invention produces a detectable color, fluorescence or UV absorbance.

The term "chemiluminescent agent" means any substance which enhances the detectability of a luminescent (*e.g.*, fluorescent) signal, for example by increasing the strength or lifetime of the signal. One exemplary and preferred chemiluminescent agent is 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and analogs. Other chemiluminescent agents include 1,2-dioxetanes such as tetramethyl-1,2-dioxetane (TMD), 1,2-dioxetanones, and 1,2-dioxetanediones.

"DNA" (deoxyribonucleic acid) means any chain or sequence of the chemical building blocks adenine (A), guanine (G), cytosine (C) and thymine (T), called nucleotide bases, that are linked together on a deoxyribose sugar backbone. DNA can have one strand of nucleotide bases, or two complimentary strands which may form a double helix structure. "RNA" (ribonucleic acid) means any chain or sequence of the chemical building blocks adenine (A), guanine (G), cytosine (C) and uracil (U), called nucleotide bases, that are linked together on a ribose sugar backbone. RNA typically has one strand of nucleotide bases.

A "polynucleotide" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") in DNA and RNA, and means any chain of two or more nucleotides. A nucleotide sequence typically carries genetic information, including the information used by cellular machinery to make proteins and enzymes. These terms include double or single stranded genomic and cDNA, RNA, any synthetic and genetically manipulated polynucleotide, and both sense and anti-sense polynucleotide (although only sense stands are being represented herein). This includes single- and double-stranded molecules, *i.e.*, DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases, for example thio-uracil, thio-guanine and fluoro-uracil.

The polynucleotides herein may be flanked by natural regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged

linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Polynucleotides may contain one or more additional covalently linked moieties, such as, for example, proteins (*e.g.*, nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (*e.g.*, acridine, psoralen, etc.), chelators (*e.g.*, metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The polynucleotides may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the polynucleotides herein may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

A "codon" is a triplet of nucleotides corresponding to an amino acid. Each amino acid is represented in DNA or RNA by one or more codons. The genetic code has some redundancy, also called degeneracy, meaning that most amino acids have more than one corresponding codon. For example, the amino acid lysine (Lys) can be coded by the nucleotide triplet or codon AAA or by the codon AAG.

The "reading frame" describes the way that a nucleotide sequence is grouped into codons. Because the nucleotides in DNA and RNA sequences are read in groups of three for protein production, it is important to begin reading the sequence at the correct amino acid, so that the correct triplets are read.

A "coding sequence" or a sequence "encoding" a polypeptide, protein or enzyme is a nucleotide sequence that, when expressed, results in the production of that polypeptide, protein or enzyme, *i.e.,* the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence. Preferably, the coding sequence is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription. A gene encoding a protein of the invention for use in an expression system, whether genomic DNA or cDNA, can be isolated from any source, particularly from a human cDNA or genomic library. Methods for obtaining genes are well known in the art, *e.g.*, Sambrook *et al.* (52).

Any animal cell potentially can serve as the nucleic acid source for the molecular cloning of the gene of interest. The DNA may be obtained by standard procedures known in the art, such as from cloned DNA (*e.g.*, a DNA "library"), from cDNA library prepared from tissues with high level expression of the protein, by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (52, 53). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

A transcriptional or translational "control sequence" is a DNA regulatory sequence, such as a promoter, enhancer, terminator, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining this invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include

the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. As described above, promoter DNA is a DNA sequence which initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. A promoter may be "inducible", meaning that it is influenced by the presence or amount of another compound (an "inducer"). For example, an inducible promoter includes those which initiate or increase the expression of a downstream coding sequence in the presence of a particular inducer compound. A "leaky" inducible promoter is a promoter that provides a high expression level in the presence of an inducer compound and a comparatively very low expression level, and at minimum a detectable expression level, in the absence of the inducer.

A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed in the periplasmic space, or outside the cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is also used to refer to a signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms. Proteins of the invention may be further modified and improved by adding a sequence which directs the secretion of the protein outside the host cell. The addition of the signal sequence does not interfere with the folding of the secreted protein, and evidence thereof is easily tested for using techniques known in the art and depending on the protein (*e.g.*, tests for activity of a given protein after modification).

Preferred signal sequences of the invention include the pelB signal sequence, which normally directs a protein to the periplasmic space between the inner and outer membranes of bacteria. Other signal sequences include, for example ompA and ompT (52). For yeast, a suitable signal sequence includes the α-subunit of *K. lactis* toxin. The signal sequence is ligated upstream of the nucleotide sequence encoding the protein, such that the sequence is present at the N-terminus of the protein after expression. Conventional cloning techniques can be used as described. Some routine experimentation within the scope of one skilled in the art may be necessary to optimize addition of the signal sequence to any given protein.

Polynucleotides are "hybridizable" to each other when at least one strand of one polynucleotide can anneal to another polynucleotide under defined stringency conditions. Stringency of hybridization is determined, e.g., by a) the temperature at which hybridization and/or washing is performed, and b) the ionic strength and polarity (e.g., formamide) of the hybridization and washing solutions, as well as other parameters. Hybridization requires that the two polynucleotides contain substantially complementary sequences; depending on the stringency of hybridization, however, mismatches may be tolerated. Typically, hybridization of two sequences at high stringency (such as, for example, in an aqueous solution of 0.5X SSC at 65°C) requires that the sequences exhibit some high degree of complementarity over their entire sequence. Conditions of intermediate stringency (such as, for example, an aqueous solution of 2X SSC at 65°C) and low stringency (such as, for example, an aqueous solution of 2X SSC at 55°C), require correspondingly less overall complementarity between the hybridizing sequences. (1X SSC is 0.15 M NaCl, 0.015 M Na citrate.) Polynucleotides that "hybridize" to the polynucleotides herein may be of any length. In one embodiment, such polynucleotides are at least 10, preferably at least 15 and most preferably at least 20 nucleotides long. In another embodiment, polynucleotides that hybridizes are of about the same length. In another embodiment, polynucleotides that hybridize include those which anneal under suitable stringency conditions and which encode polypeptides or enzymes having the same function, such as the ability to catalyze an oxidation, oxygenase, or coupling reaction of the invention.

The term "DNA reassembly" is used when recombination occurs between identical sequences. "DNA shuffling" refers herein to a group of in vitro and in vivo methods involving recombination of nucleic acid species. Such methods can be employed to generate polynucleotide molecules having variant sequences of the invention.

The term "host cell" means any cell of any organism that is selected, modified, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression by the cell of a gene, a DNA or RNA sequence, a protein or an enzyme.

The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.* for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Common expression systems include bacteria (*e.g. E. coli* and *B. subtilis*) or yeast (*e.g. S. cerevisiae*) host cells and plasmid vectors, and insect host cells and *Baculovirus* vectors. As used herein, a "facile expression system" means any

expression system that is foreign or heterologous to a selected polynucleotide or polypeptide, and which employs host cells that can be grown or maintained more advantageously than cells that are native or heterologous to the selected polynucleotide or polypeptide, or which can produce the polypeptide more efficiently or in higher yield. For example, the use of robust prokaryotic cells to express a protein of eukaryotic origin would be a facile expression system. Preferred facile expression systems include *E. coli*, *B. subtilis* and *S. cerevisiae* host cells and any suitable vector.

The term "transformation" means the introduction of a "foreign" (*i.e.* extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (*e.g.* a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (*e.g.* transcription and translation) of the introduced sequence.

Vectors typically comprise the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct."

A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, that can readily accept additional (foreign) DNA and which can readily introduced into a suitable host cell. A plasmid vector often contains

coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Promoter DNA and coding DNA may be from the same gene or from different genes, and may be from the same or different organisms. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clonetech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET or pREP plasmids (Invitrogen, San Diego, CA), or pMAL plasmids (New England Biolabs, Beverly, MA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression cassettes. Preferred vectors are described in the Examples, and include without limitations pcWori, pET-26b(+), pXTD14, pYEX-S1, pMAL, and pET22-b(+). Other vectors may be employed as desired by one skilled in the art. Routine experimentation in biotechnology can be used to determine which vectors are best suited for used with the invention, if different than as described in the Examples. In general, the choice of vector depends on the size of the polynucleotide sequence and the host cell to be employed in the methods of this invention.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, *e.g.* the resulting protein, may also be said to be "expressed" by the cell. A polynucleotide or polypeptide is expressed recombinantly, for example, when it is expressed or produced in a foreign host cell under the control of a foreign or native promoter, or in a native host cell under the control of a foreign promoter.

A polynucleotide or polypeptide is "over-expressed" when it is expressed or produced in an amount or yield that is substantially higher than a given base-line yield, *e.g.*

a yield that occurs in nature. For example, a polypeptide is over-expressed when the yield is substantially greater than the normal, average or base-line yield of the native polypolypeptide in native host cells under given conditions, for example conditions suitable to the life cycle of the native host cells. Over-expression of a polypeptide can be achieved, for example, by altering any one or more of: (a) the growth or living conditions of the host cells; (b) the polynucleotide encoding the polypeptide to be over-expressed; (c) the promoter used to control expression of the polynucleotide; and (d) the host cells themselves. This is a relative, and thus "over-expression" can also be used to compare or distinguish the expression level of one polypeptide to another, without regard for whether either polypeptide is a native polypeptide or is encoded by a native polynucleotide. Typically, over-expression means a yield that is at least about two times a normal, average or given base-line yield. Thus, a polypeptide is over-expressed when it is produced in an amount or yield that is substantially higher than the amount or yield of a parent polypeptide or under parent conditions. Likewise, a polypeptide is "under-expressed" when it is produced in an amount or yield that is substantially lower than the amount or yield of a parent polypeptide or under parent conditions, *e.g.* at least half the base-line yield. In this context, the expression level or yield refers to the amount or concentration of polynucleotide that is expressed, or polypeptide that is produced (*i.e.* expression product), whether or not in an active or functional form. As one example, a polynucleotide or polypeptide may be said to be under-expressed when it is expressed in detectable amounts under the control of an inducible promoter, but without induction, *i.e.* in the absence of an inducer compound.

An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is outside a cell. A substance is "secreted" by a cell if it delivered to the periplasm or outside the cell, from somewhere on or inside the cell.

As used herein, the terms "expression-resistant polypeptide" and "resistant to functional expression" are synonymous and refer to a polypeptide that is difficult to functionally express in selected host cells. For example, an expression-resistant polypeptide is not produced, or is produced in very low yield or in non-functional form, when a polynucleotide encoding that polypeptide is transformed or introduced into host cells, *e.g.* into a facile host cell expression system.

These polypeptides include, for example, those which have disulfide bridges, which are composed of mutiple subunits, or which require glycosylation. Expression-resistant

polypeptides also include those which are sensitive to folding and unfolding conditions, particularly intracellular conditions (inside the cell), such as temperature, pH, protein concentration, and the presence or absence of certain cofactors, coenzymes, ancillary proteins, etc. Expression-resistant polypeptides also include polypeptides that are encoded by polynucleotides which are sensitive to particular promoters or signal sequences in particular expression systems. In addition, expression-resistant polypeptides include those which tend to agglomerate, form inclusion bodies, or which are produced in a non-active or unfolded form. Particularly suitable for use as expression-resistant parent polypeptides in the invention are polypeptides that are inactive (*e.g.* they agglomerate, etc.) when produced at a high yield (*e.g.* when they are over-expressed), but which are active (*e.g.* they do *not* agglomerate, etc.) when produced at a very low yield (*e.g.* when they are under-expressed). These include, for example, polypeptides that: (a) tend to agglomerate, form inclusion bodies, or are inactive or unfolded, when expressed in the presence of an inducer, by a polynucleotide that is under the control of an inducible promoter; and (b) tend *not* to agglomerate, etc., and are active, when expressed *without* inducer, by a polynucleotide that is under the control of the inducible promoter. Such promoters are known and can be called "leaky" promoters.

Polypeptides that include, incorporate or are associated with heme groups are also examples of expression-resistant polypeptides. Particular expression-resistant polypeptides of the invention are peroxidase enzymes, such as horseradish peroxidase enzymes. An "expression-resistant polynucleotide" is a polynucleotide that encodes an expression-resistant polypeptide.

"Isolation" or "purification" of a polypeptide or enzyme refers to the derivation of the polypeptide by removing it from its original environment (for example, from its natural environment if it is naturally occurring, or form the host cell if it is produced by recombinant DNA methods). Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the protein or against

peptides derived therefrom can be used as purification reagents. Other purification methods are possible. A purified polynucleotide or polypeptide may contain less than about 50%, preferably less than about 75%, and most preferably less than about 90%, of the cellular components with which it was originally associated. A "substantially pure" enzyme indicates the highest degree of purity which can be achieved using conventional purification techniques known in the art.

The general genetic engineering tools and techniques discussed here, including transformation and expression, the use of host cells, vectors, expression systems, etc., are well known in the art.

The Screening Method.

The assay or screening method of the invention is applicable to a variety of enzymes, and is especially well suited for screening oxygenases (monooxygenases and dioxygenases) which are capable of hydroxylating a substrate.

In a broad aspect, the screening method comprises combining, in any order, substrate, oxygen donor, test oxidation enzyme, and coupling enzyme. The assay components can be placed in or on any suitable medium, carrier or support, and are combined under predetermined conditions. The conditions are chosen to facilitate, suit, promote, investigate or test the oxidation of the substrate by the oxygen donor in the presence of the test enzyme, and may be modified during the assay, for example to facilitate action by the coupling enzyme. The coupling enzyme provides a way to detect and measure successful oxidation, that is, the formation of an oxygenated product from the substrate. In some embodiments, one or more cofactors, coenzymes and additional or ancillary proteins may be used to promote or enhance activity of the oxidation enzyme, the coupling enzyme, or both.

In a preferred embodiment of the invention, test enzymes are provided by host cells which have been transformed by genetic engineering techniques, so that they express the test oxidation enzyme. The test enzyme can be produced and retained inside the cell, or it can be secreted outside the cell. In either case, test enzyme can be recovered from host cells for use in an *in vitro* or "test tube" assay, where the enzyme is combined with the other assay ingredients. Enzyme that is secreted outside the cell can usually be recovered in a non-destructive manner, by collecting it from the growth medium, usually without disrupting the cells, or on a plate where the cells are grown. When the enzyme remains inside the cell, it

is typically recovered by breaking open the cells so that the enzyme can be released and separated from the medium and cell debris.

In a more preferred embodiment, it is not necessary to recover test enzyme from host cells, because the host cells are used in the screening method, in a so-called "whole cell" assay. In this embodiment, substrate, oxygen donor, and coupling enzyme are supplied to transformed host cells or to the growth media or support for the cells. In one preferred form of this approach, the test enzyme is expressed and retained inside the host cell, and the substrate, oxygen donor, and coupling enzyme are added to the solution or plate containing the cells. Substrates, donors typically cross the cell membrane and enter the cell. If so, the substrate and donor encounter the test enzyme. Oxygenated products resulting from this encounter may cross the cell membrane (leave the cell) and react at the direction of the coupling enzyme to form a detectable reaction product. Though less desirable, any assay component which does not cross the cell membrane may be introduced directly to the interior of the cell by known means.

These techniques are particularly useful when the coupling enzyme produces a signal that can be observed from outside the cell, such as a luminescent reaction product, or when co-expression of the coupling enzyme is difficult or interferes with the reactivity of the test enzyme. Such measurements are non-destructive, and allow for isolation and further work with cells that produce active enzymes. When a fluorescent signal is used, for example, transformed host cells that produce more active oxidation enzymes "light up" in the assay and can be readily identified, and distinguished or separated from cells which do not "light up" as much and which produce inactive enzymes, less active enzymes, or no enzymes.

Oxygenated substrate that is secreted by the cell can interact with coupling enzyme in the cell media, to form a detectable extracellular reaction product. If the host cells are grown on a solid support, a fluorescent signal may be identifiable as a ring which "lights up" around cells which product active oxidation enzyme. Depending on how close together neighboring cells are growing, this method may allow for active and non-active host cells to be distinguished, but is probably less reliable than an intracellular method.

In embodiments where all of the host cells in or on a particular medium are producing the same test enzyme, the choice of intracellular or extracellular approach is likely to be determined as a matter of convenience, unless other circumstances favor or require one technique over the other.

In a particularly preferred embodiment, host cells are transformed to produce both a test enzyme and a coupling enzyme. Substrate and donor are added to the cell medium and are taken up by the cells. Active enzyme produces an oxygenated substrate, which is converted to a detectable reaction product by the coupling enzyme.

A preferred detectable reaction product is luminescent, for example fluorescent. This can be achieved, for example, by using a coupling enzyme, such as laccase or HRP, which forms fluorescent polymers from the oxygenated substrate. A chemiluminescent agent, such as luminol, can also be used to enhance the detectability of the luminescent reaction product, such as the fluorescent polymers. Detectable reaction products also include color changes, such as colored materials that absorb measurable UV light.

The method of the invention is indirect in that it does not measure the presence of an oxygenated compound which is produced by action of an oxygenase on a substrate. Instead, the invention detects or measures the reaction product that is made by the action of a coupling enzyme on a successfully oxygenated substrate. In a preferred embodiment, an oxygenated substrate is reacted in the presence of a coupling enzyme to form dimers or polymers of the oxygenated substrate. More particularly, a luminescence that is characteristic of the oxygenated substrate or its polymers is observed or measured. Most typically, the polymers are fluorescent, and can be detected by known means. This is advantageous, because oxygenated substrate may be impossible or very difficult to detect directly. For example, oxygenated substrate may not exhibit fluorescence or any other convenient marker, may do so at very low levels which are difficult to detect, or may do so at a wavelength where there are large interferences from other components of the test mixture. Thus, the invention serves to mark or amplify the oxygenated substrate or product so that is can be reliably detected or measured. The invention is sensitive to enzyme activity, and in addition is sensitive to the position of oxygenation or hydroxylation of the enzyme, *i.e.* the regioselectivity of the enzyme. For example, different colors may be produced and detected depending on where the enzyme has introduced oxygen.

A schematic representation of chemical reactions used in a preferred embodiment of the screening invention is shown in **FIG. 1**. An aromatic substrate, for example benzene, a substituted benzene or naphthalene is hydroxylated by an oxidation enzyme. Suitable enzymes include chloroperoxidase (CPO), cytochrome P450s (P450), methane monooxygenases (MMO), toluene monooxygenases, toluene dioxygenases (TDO), biphenyl dioxygenases and naphthalene dioxygenases (NDO), or any of the many mono- and di-

oxygenases.. An oxygenated product is formed, in which one or more hydroxyl (OH) groups has been substituted at one or more ring positions of the aromatic substrate, *e.g.* in place of hydrogen. These oxygenated products usually do not fluoresce, or exhibit a very small change in fluorescence, and can be difficult to detect or measure. Treatment with a coupling enzyme, such as a laccase or peroxidase (*e.g.* HRP) under appropriate conditions produces dimers or polymers of the oxygenated product which are colored or fluorescent, and can be readily detected. A chemiluminescent agent, such as luminol, can be used in addition to the coupling enzyme, to further enhance the detection and measurement of fluorescent oxygenated compounds.

Production of Test Enzymes (Host Cells and Vectors).

In one aspect of the invention, a whole cell screening method is provided, in which a test oxidation enzyme is produced by a transformed host cell using a suitable expression system. The types of host cells and expression systems which are suitable for use in accordance with the invention are those which are capable of expressing oxidation enzymes. Host cells which can also express coupling enzymes are preferred. *E. coli* is one preferred exemplary cell. Other exemplary cells include other bacterial cells such as *Bacillus, Pseudomonas*, yeast cells, insect cells and filamentous fungi such as any species of *Aspergillus* cells. For some applications, such as screening for toxicity of certain compounds, plant, human, mammalian or other animal cells may be preferred.

Suitable host cells may be transformed, transfected or infected as appropriate by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, fungal infection, microinjection, microprojectile transformation, viral infection, or other established methods. Appropriate host cells include bacteria, archaebacteria, fungi, especially yeast, and plant and animal cells. Of particular interest are *E. coli,* and *Saccharomyces cerevisiae.*

Any of the well-known procedures for inserting expression vectors into a cell for expression of a given peptide or protein may be utilized. Suitable vectors include plasmids and viruses, particularly those known to be compatible with host cells that express oxidation enzymes or oxygenases.

The invention is especially well suited for screening large numbers of mutant oxygenases wherein cells are transformed with a number of different vectors which express different mutant oxygenases. The mutant oxygenase genes can be prepared using procedures such as DNA shuffling, as shown for example in U.S. Patent No. 5,605,793 (16) or by

random mutagenesis, for example using error prone polymerase chain reactions (PCR). *See, e.g.* U.S. patent Nos. 5,741,691 and 5,811,238 (13) and PCT Application No. PCT/US98/05956 (17).

Once the host cell has been transformed with the desired vector expressing the oxygenase to be tested, the cell line is maintained and grown under conditions which promote expression of the oxygenase within the cell. In general, the oxygenase remains within the cell and is not excreted. After the transformed cells have been cultured for a sufficient time to generate oxygenase, the cells are contacted with or otherwise treated with the substrate of interest. This results in the generation of oxygenated compounds within the cell. In most cases, the oxygenated compound will diffuse from the cell where it can be reacted with a coupling enzyme to form polymeric oxygenated compounds. *See*, **FIG. 1.** Upon reaction with the coupling enzyme, the oxygenated compound forms dimers or polymers which are colored or fluorescent. The dimer or polymer is detected to provide a measure of the activity of the oxygenase. If desired, luminol or other luminescent or color enhancing material may be added to enhance the signal or provide polymers with long chemiluminescent lifetimes. Preferred cells for these applications are bacterial cells such as *E. coli* and *Bacillus*, and yeast cells, *e.g. S. cerevisiae*, in which libraries of different mutants (dozens or more, and typically thousands) can be made.

Exemplary coupling enzymes which can be used in accordance with the invention include peroxidases and laccases. Specific exemplary enzymes include horseradish peroxidase (HRP), cytochrome c peroxidase, and various other peroxidases from various microbial and plant sources such as soybean peroxidases, tulip peroxidase, lignin peroxidase, carrot peroxidase, peroxidase Novozyme® 502 , etc., as well as fungal laccase.

Although it is possible to add coupling enzyme for reaction with oxygenated compound that diffuses from the host cells, it is preferred that the coupling enzyme be co-expressed within the cells to provide an intracellular screening system. The transformation of the cell to express the coupling enzyme is accomplished in a manner similar or analogous to transforming the cell to express the oxygenase. The result is a cellular system which provides for the indirect detection of the presence of oxygenated compounds which are produced within the cell when a substrate is reacted with an oxygenase expressed within the cell. The co-expression of the coupling enzyme provides a readily available source of enzyme to polymerize the oxygenated compound to form colored, chemiluminescent or fluorescent products which can be detected within the cell.

In general terms, a preferred embodiment of the whole cell screening method includes the following steps.

1) HRP added to oxygenase-expressing cells.

Host cells that express a test oxidation enzyme are grown under conditions that will promote the functional expression of oxygenase activity. The substrate to be oxygenated is added and the oxidation reaction is allowed to proceed under appropriate conditions, *e.g.* the desired conditions (temperature, substrate, solvent, etc.) for screening which reflect the desired properties of the oxygenase. The cells can also be broken open to release the test oxidation enzyme into the medium. To detect the formation of oxygenated products, a coupling enzyme (*e.g.* a peroxidase such as horseradish peroxidase) is added to the reaction mixture (typically, the cell growth media), along with an oxygen donor, such as hydrogen peroxide. The substrate can be added before the horseradish peroxidase and peroxide, or it can be added at the same time. In some cases substrate can be added later, but this may be less efficient or otherwise less desirable. In some circumstances (e.g when the substrate is sensitive to peroxide), it is preferable to add the substrate before the other assay components. The advantage of adding substrate, oxygen donor and coupling enzyme contemporaneously is that the assay can then follow the kinetics of the oxidation reaction catalyzed by the oxygenase. The color or fluorescence, indicating the formation of an oxygenated reaction product, will accumulate in the cell culture and can be detected by any number of means. Addition of appropriate compounds (e.g. luminol) may allow the product to be detected by chemiluminescence.

2) HRP co-expressed with oxygenase (intracellular reaction).

In this embodiment, a test oxygenase and coupling enzyme (*e.g.* HRP) are both expressed by the host cell, so that coupling enzyme need not be separately added. The cells expressing both the oxygenase and HRP are grown under conditions that will promote functional expression of both activities. The substrate is added, and the reaction is allowed to proceed under appropriate conditions (desired conditions for screening). The color or fluorescence will accumulate in the cells themselves, in the cell culture, or both and can be detected by any number of means. As above, the addition of appropriate compounds (e.g. luminol) during the reaction may allow the product to be detected by chemiluminescence.

Examples of practicing the invention are provided, and are understood to be exemplary only, and do not limit the scope of the invention or the appended claims. A

person of ordinary skill in the art will appreciate that the invention can be practiced in many forms according to the claims and disclosures here.

EXAMPLE 1

Whole Cell Screening For Naphthalene Hydroxylation By Cytochrome $P450_{cam}$ With Added Horseradish Peroxidase (HRP)

This example sets forth an exemplary fluorogenic whole cell activity assay for hydroxylation of naphthalene by a mutant cytochrome P450 enzyme. This simple whole cell screening procedure avoids problems associated with assays that require disruption of cells or centrifugation steps. The example demonstrates that large libraries of enzyme mutants can be screened rapidly and effectively using the methods of the invention.

Naphthalene, an aromatic hydrocarbon, exhibits weak fluorescence. When taken up by *E. coli* host cells that express the oxygenase $P450_{cam}$, naphthalene is hydroxylated by the enzyme to produce an oxygenated product with a weak but characteristic fluorescence emission (em) at a wavelength of 430 - 465 nm. When hydroxylated naphthalene diffuses out of the cell, the $P450_{cam}$ activity is determined fluorometrically by amplifying the weak fluorescence. In accordance with the invention, HRP-catalyzed polymerization of the hydroxylated product results in a large increase in the fluorescence intensity and this is used for high throughput screening of catalysts. Although the hydroxylated naphthalene shows blue fluorescence at high concentration levels, the colonies, having a low intracellular concentration of hydroxylated naphthalene are only weakly fluorescent. With HRP-assisted fluorescence intensification, very low levels of $P450_{cam}$ activity can be detected. Therefore, there is significant benefit in terms of sensitivity to screening the enzyme mutants for improvements in activity by this method.

Cells, enzyme and chemicals. All analytical grade of chemicals were used. Horseradish peroxidase (type II, E.C. 1.11.1.7, oxidoreductase) was purchased from Sigma Chemical Co. Naphthalene and its hydroxylated derivatives, 1-naphthol and 2-naphthol, were purchased from Sigma and Aldrich. ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid] and 30% hydrogen peroxide solution were purchased from Sigma. Isopropyl-beta-d-thiogalacto-pyranoside (IPTG) was purchased from ICN Biomedicals, Inc. (Aurora, OH). Thiamine, glycerol and delta-aminolevulinic acid (ALA) were purchased from Sigma. Buffers were prepared from analytical grade reagents (pH 9: 100 mM dibasic sodium phosphate buffer, pH 7.45: 100 mM tris-HCl buffer, pH 7.0: 100 mM potassium phosphate

buffer). The *E. coli* host cells used here were strains designated as *E. coli* XL10 Gold and BL2l(DE3), obtained from Stratagene, La Jolla CA (Catalog Nos. 200317 and 200131).

Trace Element stock: 1 liter HCl solution (90% v/v distilled water: concentrated HCl) containing 0.5 g $MgCl_2$, 30 g $FeCl_2.6H_2O$, 1 g $ZnCl_2.4H_2O$, 0.2 g $CoCl_2.6H_2O$, 1 g $Na_2MoO_4.2H_2O$, 0.5g $CaCl_2.2H_2O$, 1 g $CuCl_2$, and 0.2 g H_2BO_3.

The same materials, cells, enzymes, chemicals, and trace elements stock were used, or can be used, in each of the Examples. Where significant, any differences in the subsequent Examples are noted.

A. Optimizing Expression of recombinant $P450_{cam}$ in E. coli.

An *E. coli* expression system was devised to provide host cells which are transformed by plasmid vectors containing DNA that encodes for mutant P450 oxygenases. The resulting transformants each express mutant P450 enzyme as a test oxidation enzyme for use in the invention. As shown, expression conditions were identified that reproducibly promote a high expression of $P450_{cam}$ in *E. coli.* The determination of other appropriate conditions, including selective modification of expression conditions to suit the particular needs of the assay, are well within the skill of the art.

Expression was undertaken with *E. coli* XL-10 Gold cells (from Stratagene) transformed with the expression vector pCWori(+)_$P450_{cam}$. *See*, **FIG. 2.** The plasmid backbone of pCWori + contains a pBR322 replication origin, the *lac* Iq gene, Amp^r, and a bacteriophage origin of replication. The plasmid also contains a *lac UV5* promoter and a double *Ptac* promoter region followed by a translation initiation region. The DNA sequence inserted into the plasmid backbone comprises the structural gene of $P450_{cam}$. A nucleotide sequence encoding this enzyme is set forth in **FIG. 3A [SEQ ID NO: 1].** This gene produces the native $P450_{cam}$ oxidation enzyme of *P. putida* when cloned into the *E. coli* host cell using the pCWori(+) plasmid as an expression vector. The amino acid sequence of this enzyme is shown in **FIG. 3B [SEQ. ID. NO. 2].**

Host cells transformed with this vector can serve as a control or comparison for other P450 enzymes, or other oxidation enzymes, or they can be used to produce test enzymes for use in the screening method of the invention. For example, other P450 genes, including new strains of native P450 or mutants of P450 genes may be transformed into *E. coli* host cells using the same or a similar plasmid system.

Experiments were done in both culture flasks scale and, on a smaller scale, in 96-well microtitre plates, with LB media, Terrific broth (TB), and modified minimal media (M9, containing 20% glucose or glycerol). All of these media were evaluated for induction optimization, using 1.0 mM IPTG as the inducer to activate synthesis (transcription) of the P450 oxidation enzyme in *E. coli*. For optimization of expression levels, *E. coli* XL-10 Gold ultra-competent cells (Stratagene, La Jolla, CA) transformed with pCWori+_P450cam were grown on LB/Amp plates at 37° C overnight. Single isolated colonies of transformed *E. coli* cells were then seeded into 2 ml volume of LB/amp culture media. After 8 hr growth at 37° C, an aliquot (0.5 ml) of this culture was used to inoculate a 50 ml volume of each different culture medium: LB/amp, TB/amp, and modified M9 (glucose or glycerol)/amp minimal media. One hundred microliters of pre-prepared trace element stock and 1 mM thiamine (vitamin B1) were added to each flask of the 50 ml culture media. After 8-12 hour growth (8 hours for TB, 12 hours for M9), the flask cultures were cooled to 30 °C. ALA was added (it is unstable at higher temperatures) and the cells were induced with IPTG for 24 hours.

For growth and screening in 96-well plates, one loop-full of single colonies was picked from the parent plate and directly transferred into TB/amp or M9 (glucose or glycerol) /amp media and incubated at 37°C in wells of a 96-well microtitre plate. All the additives added in the growth medium and induction conditions are the same as for the flask culture conditions described above.

$P450_{cam}$-mediated hydroxylation activity was estimated on naphthalene (NP) as the substrate. Horseradish peroxidase (HRP), as a purified form, was used as a coupling enzyme. The rate of NP conversion, which is proportional to the total amount of P450 wild-type enzyme expressed, was found to be influenced by the additives used. For example, the whole cell hydroxylation activity increased dramatically when ferrous chloride ($FeCl_2$) and thiamine (vitamin B1) were added for all media tested. At least 60 times higher activity was obtained, even in M9 minimal conditions (M9 glucose and M9 glycerol), as compared to the media which do not contain these two additives. However, addition of ALA (0.5 - 1.3 mM) resulted in a relatively small increase (20~25%) in $P450_{cam}$ activity, as compared to the thiamine and $FeCl_2$ addition, which appeared 24-48 hours into the induction period, reached a maximum at about 24 hours, and declined thereafter. Control cultures using the same plasmid pCWori(+)_$P450_{cam}$ transformed into an *E. coli* strain that received no addition of those cofactors (thiamine, $FeCl_2$, ALA) produced very little or no $P450_{cam}$ activity during at

least 48 hour culture.

The medium formulation that has been found to be the most useful for obtaining the highest whole cell activity in 96-well plates and flask cultures was Terrific Broth (TB). A 1.5-fold increase in whole cell activity was obtained when the cells were grown in Terrific broth (TB) containing 1.3 mM ALA, as compared to M9 media. One or more of these additives may be used as additives in practicing the invention, and other suitable additives may also be used in other embodiments. Reaction conditions and procedures for the whole cell activity assay on a 96-well microplate are shown in **FIG. 4A**.

B. $P450_{cam}$ purification using maltose-binding fusion affinity tag

To check whether the wild type $P450_{cam}$ can catalyze the naphthalene hydroxylation reaction using hydrogen peroxide as the oxygen donor, P450cam was expressed and purified using the maltose-binding fusion (MBP) vector pMAL-C2 from New England Biolabs (Beverly, MA). The $P450_{cam}$ gene from the pCWori(+)_$P450_{cam}$ vector was cloned into *Xmm* I and *Hind* III sites of the MBP expression vector at the 3' end of the malE-factor Xa cleavage site. The pCWori(+)_$P450_{cam}$ vector was linearized with *Nde*I (contains $P450_{cam}$ start codon, ATG), blunt-ended with Klenow (5'—3' exo-, incubated with 2.5 mM Li salt-free dTTP) and Mung bean nuclease. After the *Hind* III cut, the $P450_{cam}$ gene fragment was purified by using agarose gel extraction and then ligated to the MBP vector. The MBP expression vector contains an ampicillin marker gene and a *lacZ alpha* fragment. Transformation of *E. coli* (DH5alpha) was carried out using $CaCl_2$ and heat shock (45 seconds at 42°C). For selection of ampicillin resistance and the complete gene insert, cells grown on LB/amp agar plate was transferred to a fresh medium containing 20 ug/ml X-gal.

For $P450_{cam}$ purification, a transformant was cultivated in 500 ml TB/amp liquid medium. Except for addition of 2.35 g/l glucose, all induction and protein expression conditions were the same as described in the above in section A. (Optimizing expression of Recombinant $P450_{cam}$ in *E.coli*). Affinity separation using an amylose column was done as described by Riggs (1990) (37). The final concentration of the purified MBP-$P450_{cam}$ fusion protein (c.a. 88 kDa) was approximated by the ratio of coomassie blue dye intensities with a protein standard marker after the SDS-polyacrylamide gel electrophoresis. The final concentration of MBP-$P450_{cam}$ is estimated to be 5×10^{-8} M (Mw 89,000).

C. P450$_{cam}$ hydroxylation assay using whole cells and purified protein

The activity of P450$_{cam}$ in *E. coli* was checked by measuring the conversion of naphthalene (NP) to a hydroxylated product (e.g., 1-naphthol, 2-naphthol) which emits a blue fluorescence (lmax fl.: 465 nm with 350 nm excitation) when the exogenously added HRP polymerizes the product. The hydroxylated NP presumably diffused out of the cells, and the fluorescence was intensified by the addition of HRP and hydrogen peroxide.

Cells grown in 96-well microplates or flasks were harvested and carefully resuspended in 0.1 - 1 ml of dibasic sodium phosphate buffer (pH 9.0, 100 mM). 50 µL of this solution was then added into the same buffer (total 200 µl) containing reaction mixtures. A cell washing step is optional in both cases (however, this step reduces background fluorescence level).

Reaction conditions and procedures for the whole cell activity assay on a 96-well microplate are shown in **FIG. 4A**. In Step I, individual colonies showing fluorescence in the first screening are each loaded into a well of a 96 well plate containing 100 µL of TB media. In Step II, the colonies are allowed to grow overnight at 37° C. Then, in Step III, they are induced for 24 hours at 30° C with a 120 µL volume of IPTG and trace elements (0.5-1mM IPTG, 1 mM thiamine, 0.5-1.3 mM ALA, and 0.5 Trace Elements Stock per 10 mL of media. This induces expression of the P450$_{cam}$ enzyme. In Step IV, a test solution of substrate and oxygen donor is introduced, to provide reactants for the oxidation reaction catalyzed by P450$_{cam}$. The test solution contains:

	50 µL	culture broth (from flask or 96-well culture)
	100 µL	sodium dibasic phosphate buffer (50 mM, pH 9)
	10 µL	pure ethanol
substrate	20 µL	naphthalene stock (saturated; 1 g/13 ml in pure ethanol)
oxidant	10 µL	hydrogen peroxide stock (100 mM)
coupling enzyme	10 µL	HRP stock (1400 units/10 ml)
	200 µL	

The characteristic blue fluorescence generation inside the cells was measured in a Perkin Elmer HTS 7000 96-well microplate fluorescence reader (emission at 465 nm with excitation at 350 nm). A 96-well white microplate (Nunc, VWR) was used to reduce the background fluorescence of the reaction chamber during the detection and integration time (20 ms).

The substrate was 20 µL of a saturated solution of naphthalene (NP) in ethanol

(EtOH). The oxygen donor was a final concentration of 5 mM hydrogen peroxide (H_2O_2), and the coupling enzyme was 10 µL of HRP. The volume was adjusted to 200 µL with culture broth, buffer and ethanol. The oxygenation reaction, as an indication of $P450_{cam}$ activity, was measured using a Perkin Elmer HTS 7000 96 well microplate fluorescence reader (emission at 465 nm with excitation at 360 nm; Gain 54; measurement time 32 minutes).

The assay reaction scheme is shown diagrammatically in **FIG 4B**. A naphthalene substrate and a hydrogen peroxide (H_2O_2) oxygen donor are introduced to whole cell cultures of *E. coli* host cells transformed with pCWori(+)_$P450_{cam}$ plasmid. The plasmid contains DNA encoding the $P450_{cam}$ enzyme. The substrate and oxygen donor enter the cells, where the substrate is oxygenated in an oxidation reaction mediated by the $P450_{cam}$ enzyme. This results in oxygenation of the naphthalene substrate, to produce a hydroxylated compound or reaction product which exhibits a weak yet characteristic fluorescence. In the presence of the horseradish peroxidase (HRP) enzyme and additional hydrogen peroxide, the oxygenated compound forms a highly fluorescent polymer, which can be accurately detected.

Purified MBP-$P450_{cam}$ was also used to carry out this reaction. In this case, the naphthalene hydroxylation activities were measured in 200 µL reactions in a 96-well microplate. 5.28×10^{-9} M MBP-P450 fusion protein (one tenth dilution of the purified protein) was added to the dibasic sodium phosphate buffer containing 7 units horseradish peroxidase in purified form, and 10 mM naphthalene. Reaction was initiated after the addition of hydrogen peroxide (2.5 mM and 5 mM). The fluorescence increase (RFU, fluorescence measurement unit) was measured at the same emission and excitation using the microplate fluorescence reader.

D. Results of 96 Well Plate Assay

A screening experiment conducted on a 96 well plate is shown in tabular or chart form in **FIG 5A**. The results of this screening, using the described 96 well plate embodiment of the invention, are shown in **FIG. 5B**.

In this assay, whole cell activity for naphthalene hydroxylation by $P450_{cam}$ and hydrogen peroxide is evaluated for different media (TB, M9 glucose and M9 glycerol) with different concentrations of ALA (0.5 and 1.3 mM) in each of a series of wells on the 96 well plate. *See* **FIG. 5A**. As described above, each reaction was induced by 1 mM IPTG, 1 mM thiamine, and 0.5-1.3 mM ALA, with trace elements. Columns A-D of the 96 well plate

contained *E. coli* host cells transformed to produce $P450_{cam}$ (pCWori(+)_$P450_{cam}$ vector was used). Columns E-H contained *E. coli* cells which were not transformed to produced $P450_{cam}$ (control strain (XL-10 Gold)). Rows 1-3 of the 96 well plate contained Terrific Broth (TB) and 0.5 mM ALA. Rows 4-6 contained Terrific Broth (TB) and 1.3 mM ALA. Rows 7-9 contained M9 glucose media and 0.5 mM ALA. Rows 10-12 contained M9 glycerol media and 0.5 mM ALA. The first row of each group of three rows used 200 µL of cultivation volume. The other two rows of each group of three used 100 µL of cultivation volume.

Fluorescence in each well was measured using a microplate fluorescence reader [Perkin Elmer, HTS 7000]. The degree of fluorescence provides an indirect yet accurate indication of oxygenated substrate, which in turn provides a measure of $P450_{cam}$ activity.

As shown in **FIG. 5B**, lower P450 activity was seen for the larger, 200 µL cultivation volume that contains a smaller concentration of substrate and oxygen donor, compared to the lower (more concentrated) cultivation volume of 100 microliters. (Compare Rows 1, 4, 7 and 10 (200 µL volume) with the other Rows (100 µL volume)). This demonstrates that the observed fluorescence, and degree of fluorescence, is indeed tracking the P450 enzyme reaction and oxygenation of interest. The results also show that TB is a significantly more favorable medium than either of the M9 media tested, and the higher concentration of ALA (1.3 mM) is marginally more favorable than the lower concentration tested (0.5 mM). ALA is an important heme synthesis intermediate (P450 is a heme-protein), and the synthesis of P450 in host cell cytoplasm is regulated in part by the concentration of the synthesized heme. A high level of $P450_{cam}$ protein expression (total activity : 430 RFU/min) was obtained using TB and 1.3 mM ALA, 100 µL volume.

The experiment shows that host cells can be effectively transformed to express and active P450 enzyme which can be used to catalyze the oxygenation of a substrate in a whole cell assay adapted for high throughput screening, for example, in a 96 well plate format. The fluorescence produced by oxygenated substrate such as hydroxylated naphthalene can be reliably detected and measured, particularly when amplified by a coupling enzyme such as HRP.

To back up these results, $P450_{cam}$ peroxide-shunt pathway utilization was checked using the purified MBP-$P450_{cam}$ enzyme. Considerable increase of the poly(naphthol) fluorescence was observed: 6.8 ± 0.5 a.u. (RFU)/min/nmol with 2.5 mM H_2O_2, and 19.7 ± 0.5 a.u. (RFU)/min/nmol with 5 mM H_2O_2, in the absence of NADH and two ancillary electron transfer proteins (putidaredoxin and reductase). This supports the finding that

$P450_{cam}$ can utilize hydrogen peroxide as an oxygen donor in this reaction.

EXAMPLE 2

Whole Cell Screening For Naphthalene Hydroxylation By Image Analysis And Co-expression of $P450_{cam}$ With Horseradish Peroxidase (HRP)

This example demonstrates that co-expression of HRP with P450 monooxygenase leads to the accumulation of fluorescence inside cells, which can be monitored by digital image analysis. In EXAMPLE 1, above, HRP was added to whole cells transformed to express $P450_{cam}$. In this Example, *E. coli* host cells are transformed to express both enzymes, HRP and $P450_{cam}$. In this way, it is not necessary to add HRP in a separate assay step, nor is it necessary to monitor the growth medium for changes in fluorescence that indicate oxygenation and $P450_{cam}$ activity. In host cells transformed to produce both enzymes, the assay reaction occurs inside the cells when substrate and oxygen donor are provided, *e.g.* naphthalene and hydrogen peroxide. The fluorescence of, inside, and/or around cells that are producing an oxygenated compound and polymer (mediated by the two enzymes) can be detected and measured.

Detailed methods used in this example are given below.

A. Co-expression of recombinant HRP1A6 and $P450_{cam}$ in E. coli.

Genes and plasmids. A recombinant wild-type HRP gene that produces active HRP in *E. coli* was prepared as described in EXAMPLE 9 and in the concurrently filed U.S. application (Serial No. to be assigned), U. S. application Serial No. 09/538,591, filed March 27, 2000, and provisional application Serial No. 60/094,403 filed July 27, 1998. This HRP gene, identified as "HRP1A6", expresses enhanced amounts of HRP in E. coli, presumably due to a mutation in a non-encoding region. The gene for HRP1A6 was restricted from pETpelBHRP1A6 and cloned into the kanamycin resistant vector pET26b(+) (Novagen, Madison WI), yielding pETpelBHRP1A6Kan. Except for the antibiotic marker, this vector is identical to pETpelBHRP1A6 set forth in **FIG. 24**. Expression vector pCWori(+)_$P450_{cam}$ was prepared as set forth in EXAMPLE 1.

pCWori(+)_$P450_{cam}$ and pETpelBHRP1A6Kan transformation. Chemical transformation using $CaCl_2$ (60 mM) and heat shock (45 seconds at 42°C) was used to introduce the pETpelBHRP1A6Kan plasmid into *E.coli* BL21(DE3). Successful transformants were identified by selection on LB/kan (6-30 µg/ml kanamycin) agar plates.

Positive clones were then made chemically competent and transformed with the second plasmid, pCWori(+)_P450$_{cam}$. Identification of the *E.coli* BL21(DE3) clones containing both genes were identified by growth on LB/kan (30 µg/ml)/amp (100 µg/l amp) plates. The abbreviation "amp" indicates the antibiotic ampicillin, and "kan" indicates the antibiotic kanamycin. Cells than contain the Amp or Kan DNA fragments will grow in media that contains the respective antibiotic. This can be used as a so-called "selection marker", according to well known techniques, to identify and isolate different groups of cells with different properties using the ability or inability to resist antibiotic as a label.

B. Cell growth and reaction on agar plate for image analysis

In this procedure, pure cultures of transformed *E. coli* (containing pCWori(+)_P450$_{cam}$ and pETpelBHRP1A6Kan) were seeded onto TB/agar plates (Falcon, #1007 or Q-bot) supplemented with 100 µg/ml ampicillin, 30 µg/ml kanamycin, 100 µl/50 mL trace element stock solution, 0.25 mM thiamine, 1 mM ALA and 0.5 mM IPTG, and were grown at 37 ° C for 6 hours, at which point the incubation temperature was lowered to 30 ° C to obtain small and even colony size distribution (<0.8 mm diameter) for accurate hydroxylation activity detection. The growth temperature shifting from 37 ° C (after 6 hours) to 30 ° C is preferred for uniform cell growth control, which facilitates image analysis. It was found that cells grown at 37° C for 24 hours generally contained both smaller as well as larger cells which can not be as readily used for image analysis. After 16 hours incubation for simultaneous cell growth and protein expression, the colonies formed in the parent plates were copied (to make a replica) and transferred onto a nitrocellulose membrane, and then were incubated onto a fresh agar/M9/10 % (w/v) glucose/5 % (v/v) ethanol plate containing 6 mM naphthalene and 10 mM hydrogen peroxide for screening by fluorescence image analysis. The optimal temperature and time for this naphthalene hydroxylation were estimated to 30 °C and 12 hrs. The detailed methods are described in **FIG. 6**.

P450$_{cam}$ hydroxylation assay using whole cell co-expressing P450cam and HRP. Host cells transformed to express P450$_{cam}$ and HRP grown in 10 ml TB/amp/kan (100 µg/mL ampicillin, 30 µg/mL kanamycin) contained 0.2 mM thiamine, 1 mM ALA, and 20 µL trace elements stock solution. The grown cells were harvested and carefully resuspended in 1 ml of dibasic sodium phosphate buffer (pH 9.0, 100 mM). After the addition of 10 µl naphthalene stock (0.5 g/13 ml pure ethanol at 25°C), 10 µl ethanol, and 10 µL hydrogen peroxide solution (stock: 100 mM) to the 170 µL cell suspending solution (total 200 µl

reaction volume), the characteristic blue fluorescence generation inside the cells was measured by a Perkin Elmer HTS 7000 96 well microplate fluorescence reader (emission at 465 nm with excitation at 350 nm). A 96 well white microplate (Nunc, VWR) was used to reduce the background fluorescence of the reaction chamber during the detection and integration time (20 ms). *See*, EXAMPLE 1 and **FIG. 4A.**

HRP activity assay. The activity of peroxidase expressed in *E. coli* BL21(DE3) transformed using the vector pETpelBHRP1A6Kan, described above, was estimated colorimetrically by using ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). Cells were harvested by centrifugation (Beckman CS 6R) at 3,350 rpm and resuspended in 1 ml of 100 mM potassium phosphate buffer (pH 7.5 at 25°C). A 50 µL aliquot of this mixture was added to 40 µL of 6.4 mM ABTS solution. ABTS oxidation was monitored at 405 nm using a thermostatted spectrophotometer (Perkin Elmer UV/VIS Lambda 20) at 25°C.

C. Results of screening for co-expression of $P450_{cam}$ and HRP in E. coli.

This embodiment of the assay can be depicted as shown in **FIG. 7.** A host cell such as *E. coli* (*e.g.* strain BL21(DE3)) is transformed by two expression vectors: (1) the plasmid pCWori(+)_$P450_{cam}$; and (2) the plasmid pETpelBHRP1A6Kan. Transformed cells can be cultured from individual cells or colonies, to produce a source of transformant for use in an assay of the invention. A substrate (*e.g.* naphthalene) and oxygen donor (*e.g.* hydrogen peroxide) are introduced to the transformed host cells under favorable conditions (*e.g.* conditions which induce P450 expression and/or activity). These reactants, together with any added cofactors or coenzymes, enter each cell, where they encounter the P450 enzyme being produced there. The P450 catalyzes the hydroxylation of substrate (addition of oxygen in the form of a hydroxide group, OH) to form, for example, hydroxylated naphthalene. In the presence of a coupling enzyme also produced within the cell, such as HRP, the hydroxylated naphthalene forms oxygenated dimers and polymers which are highly fluorescent and have a characteristic fluorescence profile that can be readily detected. Typically, the polymeric oxygenated compounds do not leave the cell. Thus, the accumulation of hydroxylated product in the transformed cells provides significant advantages for detecting and measuring fluorescence, and for identifying cells which successfully produce P450 enzyme and which do so at relatively high levels. As shown coumarin, 3-phenylpropionate and other substrates may be used in place of naphthalene.

The construction of this screening system is based on inducible prokaryotic expression vectors that allow the active co-expression of both enzymes, $P450_{cam}$ and HRP, in the same host strain. As described, a pCWori+ vector which contains the $P450_{cam}$ gene was inserted in *E. coli* BL21(DE3). This results in *E. coli* host cells that express the introduced P450 gene, and produce a functional P450 enzyme that can be used to catalyze the first reaction in the assay, the oxygenation reaction described above. For the second enzymatic reaction, the coupling reaction, an active HRP gene inserted into a pET26b expression vector was also transformed into *E. coli* BL21(DE3) as described above. Alternatively, cytochrome c peroxidase (CCP) can be used as the coupling enzyme, and a functional gene expressing this enzyme can be transformed into *E. coli* or into a yeast host cell using similar means. Yeast can also be used as the host cell for expression of P450 enzymes, or co-expression of oxygenase and coupling enzyme. Characteristics of these expression vectors are summarized in TABLE 1.

TABLE 1

Characteristics of plasmids used for coexpression of P450cam with HRP and CCP coupling enzymes.

Gene insert; vector	Promoter type	Replication origin	Antibiotic marker	
P450cam from *P. putida* (ATCC17453); pCWori+	Ptac Ptac	PBR322	Amp^r	* RBS and I.C. spacing : 3bp
HRP1A6; pET-26b(+)	T7	PBR322	Kan^r	pET-26b(+) contains pelB leader
Cytochrome c peroxidase from *S. cerevisiae* ; pET-26b(+)	T7	PBR322	Kan^r	** no pelB leader

* 3 bases spacing between ribosomal binding site and initiation codon.

** pelB leader sequence was removed from the original pET-26b(+) vector to avoid protein secretion.

The experimental time course of the fluorescent product generation using this co-expression system is illustrated in **FIG. 8**. With the HRP/$P450_{cam}$ double vector system, a

more than 370% increase in absolute cell fluorescence level was observed after the 30 minute reaction with 5 mM hydrogen peroxide.

A study of the effects of IPTG concentration over the range 0-1 mM indicated that 0.5 mM IPTG is optimal for the coupled $P450_{cam}$ /HRP *E. coli* BL21(DE3) co-expression system used in this example. As shown in **FIG. 9**, this concentration of IPTG induces the highest activity for P450 enzyme in the presence of an appropriately high HRP activity. Thus, co-expression of $P450_{cam}$ and HRP at appropriate levels (IPTG ~0.5 mM) resulted in marked intensification of intracellular fluorescence level.

This co-expression system is advantageous in that the fluorescence remains associated with the cell (nondiffusible). The background intensities either remained constant with time (host strain as a negative control) or showed small increases with time (cells without naphthalene or hydrogen peroxide). As a result, intracellular HRP expression with $P450_{cam}$ activity in BL21(DE3) was shown to be an effective self-contained and complete screening system for detecting hydroxylation reactions which utilize the peroxide-shunt pathway. Although $P450_{cam}$ itself also produces fluorescent naphthols, the total intensity measured was lower than the HRP/$P450_{cam}$ co-expression system.

D. Image Acquisition, Processing and Analysis

Images of whole cell fluorescence on agar plates were scanned using Eagle Eye II and a top-mounted 350 nm ultraviolet illuminator (Stratagene, La Jolla, CA). Images were digitally analyzed using the software package Optimas 5.0 (Optimas Corporation, WA). Gray-level fluorescent colonies were filtered using a blue fluorescence band-path filter with excitation at 350 nm to remove background fluorescence. Setup parameters for the acquisition of the fluorescent signals using this BL21(DE3) system are as follows: blue band-pass filter (430-470 nm range), lens zoom level = 4 x, fluorescence image exposure time = 1/10 second. Selected gray-level colonies were analyzed with a charge-coupled device (CCD) and subsequent computer-assisted image analysis. A weighted score of 255 was used, with zero as the bottom value and 255 as the highest fluorescence intensity. (This configuration can be modified as appropriate.)

The background mean averaged fluorescence intensities of the host *E. coli* strain BL21(DE3) (plasmid-free control strain) were estimated to be 0 to 5. Fluorescence intensities were calculated based on the 27,000-grade scale. The fully automated image

segmentation algorithm (pattern recognition and back-propagation algorithm) for colony recognition and size measurement was adapted to avoid time-sensitive and subjective manual tracing of colony contours. Individual colony size measurement and automatic single isolated colony detection were derived from computer-determined colony boundaries and fluorescence differences with different sets of threshold levels. The estimated total analysis time was about 5 seconds for 10^5 colonies.

Blue fluorescence is derived from the products synthesized by the coupled enzymatic reactions catalyzed by $P450_{cam}$ and HRP. The scanned fluorescent images gave a clear result which is well correlated with the specific $P450_{cam}$ hydroxylation activity. It is suggested that smaller individual colonies are better for fluorescence image analysis. The maximum size limit of the colony for this image analysis was estimated to be 0.8 mm diameter. Typical dimensions of the imaged colonies were about 0.4 to 0.8 mm in diameter, and there were approximately 9 to 17% fluorescence value deviations within this size distribution.

Scanned images (**FIG. 10**) were further processed by configuring overall thresholding, geometry recognition, intensity quantification, global and local segmentation, and cutting edge to reduce background fluorescence. A main consideration was the separation of the overlapping colonies in the two-dimensional cell fluorescence image. The original fluorescence image scanned was rather complex and involved many unclear edge cuts to analyze (**FIG. 11A**). By imposing the sequential combinations of Boolean bit-map digitization and by passing through a uniform luminance enhancement algorithm (Extensis, Extensis Co.), the images could be fine-tuned for further evaluation (mainly, cutting edge by volume downsizing, boundary deletion, and dividing) in the OPTIMAS analyzer. *See* **FIGS. 11B-11D**. During the second image processing, colonies touching each other were first selected for semi-automatic algorithm provided by this package and then a boundary detection algorithm was run to delete any colonies that hit the boundary. **FIG. 11B** still exhibits the regions of two or three cells in contact, and **FIGS. 11C** and **11D** show improved fluorescence images after several cycles of processing.

Using these image analysis techniques, **FIG. 12** shows a comparison of fluorescence intensities in *E. coli* BL21(DE3) for the following combinations:

A. *$P450_{cam}$/HRP* co-expression with addition of naphthalene and H_2O_2;

B. $P450_{cam}$ with addition of naphthalene and H_2O_2;

C. HRP with addition of naphthalene and H_2O_2

D. Untransformed *E. coli* BL21(DE3) host strain as a negative control;

E. $P450_{cam}$/HRP co-expression with H_2O_2 and without naphthalene;

F. $P450_{cam}$/HRP co-expression with naphthalene and without H_2O_2.

Left side images are 2-dimensional original fluorescent colonies scanned. The right side histograms, moonscape view, are the resulting fluorescence intensities of the individual colonies.

Co-expression of $P450_{cam}$/HRP (Combination A) gave the highest fluorescence intensity among the cells tested. There was a 3-fold increase of the absolute fluorescence level between the cells harboring $P450_{cam}$/HRP vectors (A) and $P450_{cam}$ expression vector alone (B), as estimated in the moonscape view. Due to the low level of the fluorescence generated, approximately less than one-quarter of the colonies could be counted with the cells harboring only the $P450_{cam}$ expression vector. The background fluorescence levels tested with the other four cases (HRP, BL21(DE3) host strain, and in the presence or absence of naphthalene and H_2O_2) were much lower and clearly distinguishable from the fluorescence generated by the co-expression system. Scored fluorescence intensities of these control cases (**FIGS. 12C, 12D, 12E,** and **12F**) almost all fell between 0 to 5. None of these four cases (C, D, E, F) scored a hit during the image analysis. Therefore, cells expressing the oxygenase and peroxidase enzymes can be identified by plate-based image analysis as active in the hydroxylation reaction.

FIG. 13 shows a technique for automatically detecting fluorescent colonies and the fluorescence intensity analysis result. A total of 843 cells in a 5 x 5 sq. cm scanned area were counted (out of 20,000 colonies counted in an entire 25 x 25 sq. cm plate). The individual positive colony fluorescence intensities could be integrated in the scanned area. The *E. coli* cells exposed to naphthalene on the plate survived during the 24 hours incubation. The computer-assisted techniques described above may also be used in connection with an automated or high speed embodiment.

The image analysis results are consistent with the data obtained from assays carried out in 96-well plates (*see* **FIG. 8**). In these experiments it is shown that the cells having $P450_{cam}$ alone resulted in relatively low fluorescence formation (almost three times lower absolute fluorescence level), as compared to the two-enzyme approach. Even though the naphthols exhibit fluorescence, the intensities estimated were very low

at nanomolar concentration levels (200 - 250 a.u. for 10 - 100 nmol/ml). Thus, direct whole cell fluorometric observation of the $P450_{cam}$ hydroxylation reaction can be realized, and co-expression with HRP leads to significant advantages for screening.

An important advantage of this enzyme-coupled assay system is that it generates an amplified fluorescence signal proportional to the formation of oxygenated product. This intensified signal allows the screening of large numbers of host cells expressing oxygenase enzymes, for example, by fluorescence digital imaging or by fluorescence activated cell sorting (FACS). The greater the signal amplification provided by the use of the coupling enzyme, the lower the oxygenase activity that can be identified by this screening process. Furthermore, fewer false positives and false negatives will be identified during screening for improved oxygenases.

EXAMPLE 3

Whole Cell Screening For Cytochrome P450 Activity Towards Other Substrates By Image Analysis And Co-expression of $P450_{cam}$ with Horseradish Peroxidase (HRP)

In this example coumarin and 3-phenylpropionate are used as substrates in place of naphthalene. Co-expression of HRP with the P450 monooxygenase leads to fluorescence generation for coumarin and 3-phenylpropionate as substrates of the hydroxylation reactions. All experimental conditions are the same as those described in EXAMPLE 2, except for the substrate concentrations used. The final concentrations of 3-phenylpropionate and coumarin were 1.2 g/l and $6x10^{-2}$ g/1, respectively.

Coumarin and its hydroxy derivatives, 7-hydroxycoumarin and 4-hydroxycoumarin, were purchased from Sigma Chemical Co. (St. Louis, MO). 3-phenylpropionate and its 2-/4-hydroxy derivatives (3-(2-hydroxyphenyl) propionate and 3-(4-hydroxyphenyl) propionate) were also purchased from Aldrich and Sigma Chemical Co. Characteristic hydrocoumarin or hydroxy (3-phenylpropionate) peaks were detected using a fluorimeter with a broad band-path fluorescence emission filter (465 +/- 30 nm); *i.e.* a Perkin Elmer HTS 7000. The whole cell reaction system using co-expression of $P450_{cam}$ with horseradish peroxidase was used. At pH 9, 4- and 7-hydroxycoumarin also show a characteristic blue-green fluorescence (emission at 450-495 nm; excitation at 350 nm). This fluorescence is hardly detectable, however, because coumarin itself also exhibits quite strong fluorescence, leading to high background fluorescence under these conditions.

FIG. 14 shows the results of the HRP-assisted fluorescence intensification for two substrates, coumarin (**FIG. 14A**) and 3-phenylpropionate (**FIG. 14B**). Each numbered bar graph shows, respectively, the results of: (1) $P450_{cam}$ and HRP1A6 co-expression in *E. coli* BL21(DE3) host cells; (2) $P450_{cam}$ expression in *E. coli* BL21(DE3) host cells; (3) HRP1A6 expression in *E. coli* BL21(DE3) host cells; and (4) *E. coli* BL21(DE3) host cells. With coumarin, which is already quite fluorescent, some intensification was found: 35% higher than with the $P450_{cam}$ expression alone. In the case of 3-phenylpropionate, HRP assistance gave 300% higher fluorescence intensity, as compared to the controls ($P450_{cam}$ in BL21(DE3), HRP in BL21(DE3), and the host strain).

EXAMPLE 4

Hydroxylation assay based on chemiluminescence light enhancement

This example demonstrates the use of chemiluminescence detection for monitoring the formation of hydroxylated products, using horseradish peroxidase as the coupling enzyme. *See* **FIG. 15**. In this example, the coupling enzyme is coexpressed with the oxidation enzyme in bacterial cells, as shown for example in EXAMPLE 2. In a 96 well plate assay, as previously described, the signal afforded by using a P450 monooxygenase and the HRP coupling enzyme was measured (column 4 in **FIG. 15A**) and compared to the signal from cells that do not have the coupling enzyme (column 5), do not carry out the hydroxylation reaction (column 6) and carry out neither reaction (column 7). Rows E and F contain the substrate (3-phenylpropionate), while rows G and H contain no substrate.

The oxidation of the chemiluminescent agent luminol by peroxidase catalysis leads to a colored product, but generates no (or very weak) chemiluminescence in the presence of just hydrogen peroxide (no coupling enzyme). The photocurrent (intensity) of the generated chemiluminescence light was measured using an AlphaImager system (AlphaImager 2000, ver. 3.3, AlphaImager Corporation). This luminometer includes a multichamber cabinet for luminescence detection (a light-tight box with a matte-black interior), photocurrent analysis software (AlphaImager 2000, ver. 3.3), and a CCD detector for light intensity detection. Light emitted from individual wells of a 96-well type white "Nunc" fluoroplate was measured in the camera luminometer.

Luminol was purchased from Molecular Probes (Eugene, OR), and 3-phenylpropionate and 30 % hydrogen peroxide were purchased from Sigma. The reaction mixture (200 ul) contained 0.1 mM borate buffer 8.6 with sodium perborate (3 mM),

luminol (60 and 120 uM), and 3-phenylpropionate (0.5 mM). Cell growth and $P450_{cam}$/HRP co-expression conditions are the same as in EXAMPLE 2. After the 24 hour induction to produce the $P450_{cam}$, hydroxylation of 3-phenylpropionate was carried out for 20 min. The hydroxylation reaction conditions are as described in EXAMPLE 2. Hydrogen peroxide (5 mM) was added.

Reaction conditions and results are shown in **FIG. 15**. The luminescence measurements show that the P450cam-catalyzed hydroxylation of the 3-phenylpropionate enhances the luminescence light generation. The chemiluminescence of the cells containing expressed P450cam and HRP (Lane 4, Row F of **FIG. 15B**) was enhanced up to 98-fold, as compared to the luminol reaction itself (Lane 4, Rows G and H). The hydroxylated 3-phenylpropionate therefore leads to a significant increase in the light emission and can be monitored using this approach. The integrated light emission of the strain coexpressing both enzymes shows more than 1000-fold increase in the first 40 seconds after the reaction is initiated, as compared to other background levels. The intense and prolonged light emission from the reaction enhanced by the incorporation of the additional hydroxylated aromatic phenol lasted more than 7 minutes. This is particularly useful for the screening of enzymes with relatively weak hydroxylation activities, for example in connection with particular substrates. Moreover, multiple colonies can be assayed rapidly and simultaneously by using colony image analysis. The abbreviation "ILDV" (*e.g.* **FIG. 15B**) indicates the integrated light density value, as a chemiluminescence intensity unit. The results in epi UV conditions show that the 3-phenylpropionate hydroxylation can also be detected using the combined forms of light intensities (a kind of light energy amplification), which were generated by chemiluminescence and fluorescence-like light emission, separately (**FIG. 15A**). Although the absolute light density was increased, in this case, the dual mode detection gained increased background.

EXAMPLE 5

Monitoring oxidation by co-expression with cytochrome c peroxidase

This example demonstrates the use of another peroxidase, cytochrome c peroxidase (CCP), a gene from yeast that is expressed in *E. coli* as the coupling enzyme for screening $P450_{cam}$-catalyzed hydroxylation. The yeast CCP enzyme is expressed in functional form in *E. coli* host cells.

A. Cytochrome c peroxidase vector construction

The *S. cerevisiae* cytochrome c peroxidase (CCP) gene from pT7CCP (donated by Dr. David Goodin, The Scripps Research Institute, La Jolla, CA) was recloned into *Nde* I and *Bam* HI sites of kanamycin resistant pET-26b(+) expression vector (purchased from Novagen, Inc., Madison. WI). pT7CCP carries a gene for CCP in which the N-terminal sequence has been modified to code for amino acids Met-Lys-Thr, as described in Goodin et al. (1990) (39), and Fitzgerald et al. (40). First, the pT7CCP vector containing the CCP gene was linearized with *Pvu* II, blunt-ended with Mung-bean nuclease to give a ligation site between the 3'-end of this gene and the *Bam* HI site in pET-26b(+). Next, this gene fragment was cut using *Nde* I for 5'-end ligation with the vector. The N-terminal pelB signal sequence which is located in the upstream region (224-289) of the pET-26b(+) vector was removed by *Nde* I and BamH I digestion, to allow intracellular CCP expression. *Bam* H I cut was then blunt-ended for further ligation with the engineered CCP gene fragment. **FIG. 16** shows the pET26 b+CCP vector map.

B. Whole cell screening for cytochrome P450 activity using CCP co-expression

Expression was undertaken with *E. coli* BL21(DE3) cells transformed with pCWori(+)_$P450_{cam}$ and pET26 b+CCP vectors, as previously described. The plasmid backbone of pET-26b(+) contains a T7 promoter at 361-377 region, f1 plasmid, and a kanamycin coding region. Cell growth and assay conditions are the same as EXAMPLE 2 except for inducer and kanamycin concentrations. For the co-expression of both enzymes, $P450_{cam}$ and CCP, 1 mM IPTG and 50 ug/ml kanamycin were used. The results are shown in **FIG. 17**. Co-expression of P450cam/CCP gave highly intensified fluorescence signals when naphthalene hydroxylation was tested. A 3.2-fold increase of the absolute fluorescence level, as compared to P450cam catalyzed reaction alone, was observed.

EXAMPLE 6

Use Of Laccase As Coupling Enzyme

In this example, a laccase enzyme was used as a coupling enzyme, instead of a peroxidase. When a laccase is used, there is no need to add hydrogen peroxide, as this enzyme can catalyze the oxidative coupling reaction using molecular oxygen. This is useful when screening oxidative enzymes that do not require peroxide for the reaction.

Laccases are copper-containing enzymes that catalyze the oxidation of a variety of

substrates, such as phenols, mono-, di-, and poly-phenols, methoxy-substituted phenols, and aromatic amines. Laccases couple four of these one-electron oxidations to the irreversible four-electron reduction of dioxygen to water. Each one-electron reaction generates a free radical. Aryloxy radicals formed by laccases are unstable and typically undergo a second reaction. This reaction may be a second enzymatic oxidation (converting phenol to quinone in many cases), a nonenzymatic reaction such as hydration, disproportionation, or oxidation/reduction, or the radical may couple to other phenolic structures in a polymerization reaction that produces products that are often colored and/or highly fluorescent.

Laccase was purchased from Sigma Chemicals as a crude acetone powder from the fungus *Rhus vernificera*. The laccase powder had a minimum of 50 units per mg., with unit activity defined as) A_{530} of 0.001/min. at pH 6.5, 30°C, 3 mL solution with syringaldazine as substrate. Type II HRP (RZ approximately 2.0) and all other chemicals were purchased from Sigma. Fluorescence readings were taken with a Perkin-Elmer HTS 7000 plate reading fluorimeter. Excitation and emission wavelengths were 360 nm and 465 nm, respectively.

Experiments were performed at 25° C in the wells of opaque, white Nunc 96-well plates with a total liquid of 200 uL. Except where stated otherwise, each well contained 10% pure ethanol to improve the solubility of substrates and products. Where HRP was used for comparison, approximately 2.3 units of HRP and 5 mM H_2O_2 were in each well. Each experiment was performed at pH values of 6.5, 7.5, and 9.0 using phosphate buffers (10 mM and 100 mM, depending on the experiment), which shows no fluorescence. Tris buffer was not used because it increases background fluorescence. Except where stated, results are given from conditions at pH 9.0, which were either the best results or barely distinguishable from the other conditions.

In order to evaluate laccase in a useful whole cell assay to identify the formation of hydroxylated aromatic compounds by oxidative enzymes (such as cytochrome $P450_{cam}$ and toluene dioxygenase), laccase was added to solutions containing cells, naphthalene, and naphthol. $P450_{cam}$ was expressed in *E. coli* strain BL21(DE3) using plasmid pCWori+. BL21(DE3) cells with and without the expressed protein were grown in Terrific Broth and after 8 hours were induced with 1 mM IPTG for 24 hours. 50 µL of each type of cell solution (with or without plasmid) was added to twelve wells (six wells for each type). pH 9 buffer was added to each well so that the final volume after all additions would be 200 uL. Approximately 15 units of laccase was added to each well, and the mixtures were allowed

to pre-incubate for about 45 minutes to remove any high background activity between laccase and cell solution components. In one set of six wells, three with plasmid-harboring cells and three with non-transformed cells, 10 μL of naphthalene saturated in ethanol was added to each solution. 1-Naphthol was then added to one of each type of well (with and without naphthalene; and with and without $P450_{cam}$) to a concentration of 100 uM. Similarly, 2-naphthol was added to four other wells. These wells in which naphthol was added (in addition to naphthalene) simulate situations in which $P450_{cam}$ is capable of producing naphthol from naphthalene at high concentrations (100 uM). All wells contained 10% ethanol. For comparison, the same twelve wells were prepared using HRP and H_2O_2 instead of laccase. These same experiments were performed at pH 6.5.

In all wells containing 1-naphthol (100 uM) and either HRP or laccase a color change from the light yellow of the cell solution to a dark brown color occurred. The change was more rapid with HRP (approximately 1 minute compared to approximately 1 hour). In the case of 2-naphthol, color change to a light orange occurs, although this is less pronounced and slower. The difference in color formation between comparable solutions may produce stronger color changes, in theory because the HRP preparation itself already has a slightly brown color. It is relatively difficult to discern a difference in color between the comparable solutions with and without naphthalene added or with and without P450 expression, indicating that the level of naphthalene hydroxylation by the enzyme is very low at the expression level in this experiment, and in its activity towards this substrate under the test conditions. No color change was observed for any of the wells not containing naphthol. These results indicate that as long as naphthol is produced in a high enough concentration by the hydroxylating enzyme, laccase can be used as a coupling enzyme for naphthol identification in a colorimetric whole cell assay.

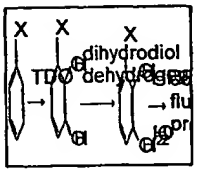
EXAMPLE 7

Detection of Catechols Formed by Toluene Dioxygenase (TDO)-Catalyzed Dioxygenation of a Substituted Benzene

This example demonstrates the use of horseradish peroxidase for detecting the formation of the products (catechols) of TDO-catalyzed dioxygenation of chlorobenzene followed by dehydrogenation. A host cell, *E. coli* in this example, is transformed with a vector having a functional TDO gene, and transformed cells are grown under conditions suitable for TDO expression. Host cells in this example are also transformed to express the

enzyme dihydrodiol dehydrogenase, and they may be transformed to express HRP, as described in other EXAMPLES herein.

The overall reaction used in the assay of this example is shown below.



The first set of reactions (catechol formation) is catalyzed by *E. coli* DH5 alpha containing plasmid pXTD14, which contains the genes *todC1C2BA* (for toluene dioxygenase) and *todD* (for dihydrodiol dehydrogenase). A map of this construct is shown in **FIG. 18**.

For plasmid construction, *E. coli* DH5 alpha was used as a host and the transformants were grown in LB containing 50 µg/ml ampicillin at 37 °C. The *E. coli* expression vector pTrc99A was purchased from Pharmacia Biotech (Uppsala, Sweden).

A 2.1 kb wild type *todC1-todC2* fragment was produced by PCR on template pDTG601 (provided by D. Gibson, University of Iowa) (41), using the following primers: a forward primer TDO-5F: 5'-GATCATGAATGAGACCGACACATCACCTATC-3' **[SEQ ID NO: 3]**; and

a reverse primer TDO-2R: 5'-ACGAATTCTAGAAGAAGAAACTGAGGTTATTG -3' **[SEQ ID NO: 4]**.

The fragment was digested with *Bsp*HI and *Eco*RI and subcloned in *Nco*I-*Eco*RI site of pTrc99A to construct pXTD2. Restriction sites in the primers are underlined.

A DNA fragment containing *todC2-todB-todA-todD* genes was amplified from pDTG602 (provided by D. Gibson, University of Iowa) (41) by PCR using the following primers:

a *Bam* HI-tagged forward primer TDO-9F (*Bam* HI restriction sequence is underlined),

(5'-TTGGATCCGGTGGACCTTGTCCATTTG-3' **[SEQ ID NO: 5]**; and

a reverse primer TDO-14R (*Xba* I restriction sequence is underlined)

(5'-GCTCTAGATCAACCGAAGTGCTTGTCGAG-3' **[SEQ ID NO: 6]**.

The resulting 3.0 kb fragment was digested with *Bam* HI and *Xba* I, and purified by QIAquick PCR Purification Kit (QIAGEN). This fragment was cloned in *Bam* HI-*Xba* I site of pTrc99A to yield plasmid pXTD10. Then pXTD10 was digested with *Eco*RI and *Bam*HI and ligated to a 1.2 kb wildtype *todC1* fragment digested with *Eco*RI and *Bam*HI. This wild type *todC1* fragment was produced by PCR using:

a forward primer TDO-12F: 5'-CGGAATTCTAGGAAACAGACCATG-3' **[SEQ ID NO: 7]**; and

a reverse primer TDO-13R: 5'-CCGGATCCAACCTGGGTCGAAGTCAAATG-3' **[SEQ ID NO: 8]** from template DNA pXTD2. Restriction sites in the plasmids are underlined. The resulting plasmid is pXTD14. *E. coli* strain DH5 alpha transformed with pKK223-3 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as a control.

In this example, a chlorobenzene substrate is oxygenated by the addition of two hydroxyl groups, via TDO, and the ring structure of the substrate is stabilized to a double bond via dihydrodiol dehydrogenase. The oxygen donor in this reaction is molecular oxygen (O_2), obtained by the *E. coli* host from O_2 dissolved in the medium. In another reaction, the dihydroxylated product is reacted in the presence of HRP and hydrogen peroxide, to form colored or fluorescent products. Thus, in this example, the substrate is chlorobenzene (or any suitable aromatic substrate), the oxidation enzyme is toluene dioxygenase (TDO), the oxygen donor is molecular oxygen, and the coupling enzyme is horseradish peroxidase (HRP).

The following procedure was used to prepare supernatant containing TDO-produced catechol:

1) Add 0.5 µL of each overnight seed culture to two flasks containing 20 mL of LB-Amp and shake for three hours at 37 °C.

2) Add 200 µL of 100 mM IPTG to each flask, and shake at 30° C for two hours.

3) Centrifuge the cultures at 3000 rpm for 10 minutes and discard the supernatant.

4) Resuspend the pellet in 4 mL of 50 mM phosphate buffer, pH 7.4, containing 10 mM chlorobenzene and 0.2% glucose.

5) Incubate at 30° C for two hours (2 mL in 15 mL tube).

6) Add 12 mL of 50 mM phosphate buffer, pH 7.4, and centrifuge at 3000 rpm for 10 minutes.

7) Transfer catechol-containing supernatant to a fresh tube.

To detect the catechol products, 10 µL of 2 mg/mL HRP and 10 µL of 1 M H_2O_2 was added to 200 µL of supernatant. A two times dilution of the supernatant was also analyzed. In the case of *E. coli* containing pXTD14, the solution turned red shortly after addition of HRP and H_2O_2 to the catechol-containing supernatant. The 2X dilutions were subjected to spectro-photometric analysis. The baseline was taken to be the control cultures (pKK223-3) supernatant with only H_2O_2 added. The absorbance profile of the pKK223-3 with HRP and H_2O_2 was essentially flat. The absorbance profile of the TDO-expressing strain (pXTD14) showed a small peak at 281.5 nm which, on the basis of previous experiments, corresponds to the presence of chlorocatechol. When HRP was added to the supernatant from pXTD14, absorbances appeared around 340 nm and 500 nm that correspond to the polymers formed when the chlorocatechol is oligomerized by HRP.

EXAMPLE 8

Identification of improved mutants of $P450_{cam}$

An important aspect of this invention is to identify mutants in a high throughput screen of mutagenized gene libraries. A screening strategy with high throughput fluorescence image analysis has been implemented, in order to identify bacterial clones expressing improved hydroxylating enzymes. Mutants of $P450_{cam}$ with improved activity on naphthalene and hydrogen peroxide (peroxide shunt pathway) have been identified. These mutants are also more active on a related substrate, 3-phenylpropionate.

In general, the method uses polymerase chain reaction (PCR) techniques to generate a library of oxygenase mutants, using DNA sequences (*e.g.* as primers and/or probes) from a known or starting enzyme as a template. In this example, mutants of $P450_{cam}$ were derived from the $P450_{cam}$ gene discussed above.

A. P450cam gene mutagenesis

The mutagenic PCR protocol of Cadwell and Joyce (1992) (15) was used with some modifications. For a 100 µl reaction, the following were included:

10 µl 10 X buffer (Boehringer Mannheim, Germany; PCR reaction buffer)

100 mM Tris/HCl, 500 mM KCl, pH 8.3 at 20 °C)
28µl $MgCl_2$ (25 mM stock solution)
0.2 µl dATP (100 mM stock)
0.2 µl dGTP (100 mM stock)
2 µl dCTP (100 mM stock)
1 µl dTTP (100 mM stock)
0.7 mM $MnCl_2$
1.5 µl forward primer (9.8 pmol/ul)
1 µl reverse primer (14.0 pmol/ul)
1 µl (5 unit) *Taq* polymerase (Boehringer Mannheim)
0.01 % gelatin (from 10x stock)
20 fmoles of template pCWori(+)_$P450_{cam}$
42.1 µl ddH_2O.

Error-prone PCR was performed in a programmable thermocycler (PTC200, *MJ* Research) for 30 cycles. (denaturation 94 °C, 30 s; annealing 45 °C, 30 s; elongation 72 °C, 2 min). The forward (24-mers) and reverse primer (25-mers) sequences used were:

[SEQ ID NO: 9] 5'-CATCGATGCTTAGGAGGTCATATG-3', and

[SEQ ID NO: 10] 5'-TCATGTTTGACAGCTTATCATCGAT-3',

where the *Nde* I restriction site is underlined. The total insert gene size to be amplified between two primers is 1.4 kb.

B. DNA purification, cloning and expression

The Qiaex II kit (Qaigen, Germany) was used for PCR product purification. Purified PCR product was redissolved in TE buffer (10 mM Tris-HCl, pH 8.0) and was subjected to electrophoresis on preparative 1 % agarose gels to check the purity. After digestion with *Nde* I (10 u) and *Hind* III (10 u) for 2 hours at 37 °C, the *Nde* I-*Hind* III fragment was purified again by gel extraction and was inserted into pCWori+ shuttle vector. The ligation was carried out at 16 °C for 9 hours with 200 U of T4 DNA ligase (Boehringer Mannheim). The ligation mixture was then used to transform *E. coli* BL21(DE3) Gold cells (Stratagene) which also have pETpelBHRP1A6Kan introduced as described in other examples herein.

For selection of the cells containing two different plasmids, a TB/amp(100 ug/ml)/kan(30 ug/ml) plate was used for cell growth and simultaneous protein expression.

The *E. coli* strain containing pCWori(+)_P450$_{cam}$ and pETpelBHRP1A6Kan was grown at 37 °C for 6 hours, then was induced for P450$_{cam}$ and HRP expression by shifting the incubation temperature to 30° C. After 16 hours, the colonies were stamped onto nitrocellulose membranes and transferred onto fresh plates containing naphthalene and hydrogen peroxide for fluorescence image analysis, using the protocol of EXAMPLE 2.

C. Results of Screening for Mutant P450 Activity in a Whole Cell System

Approximately 55,000 mutant P450$_{cam}$ clones on 3 Q-bot plates were screened on naphthalene as a substrate using fluorescence digital imaging. Selected highly fluorogenic mutant colonies identified by digital imaging were transferred to a 96-well plate for confirmation by more detailed measurements, as described in EXAMPLE 2.

FIG. 19A shows the results of a digital scan of sections of plates containing fluorescent mutant P450$_{cam}$ colonies. The colony fluorescence values are plotted in descending order. Adjusting the threshold level to the point where the wild type fluorescence is near or lower than the detection limit allows one to see (count) only the colonies expressing P450$_{cam}$ activity comparable to or greater than wild type levels. This demonstrates one of the advantages of using imaging methods in screening, as compared to, for example, assays in microtitre plates. In the microtitre plates inactive or poorly active clones must be counted (measured) alongside active ones.

A large number of the colonies (about 20%) show activity roughly comparable to or higher than wild type P450$_{cam}$ activity. The wild type level is about 320 fluorescence units. The highest mutant activity showed 1830 fluorescence units, a nearly six-fold increase in fluorescence.

FIG. 19B shows the results of scanning about 200,000 mutants. Fluorescence values of about 32,000 of the clones are plotted in descending order. Three mutants having a high activity compared to wild-type P450$_{cam}$ are indicated. These three clones with enhanced fluorescence were selected for growth and confirmation of the enhanced activity towards naphthalene in a whole cell assay. The fluorescence over time of each of these three mutants and wild-type is shown in **FIGS. 19C** (wild-type), **19D** (Mutant M7-4H), **19E** (M7-6H), and **19F** (M7-8H). Clone M7-6H showed an 11-fold increase in activity as compared to wild type P450cam. Two other clones (M7-4H and M7-8H) identified by the digital image scanning also showed improved activity on this substrate, with the largest increase of 3.2 fold for M7-6H.

For comparison, 96 randomly selected clones from a large mutant library (about 20,000 colonies) were assayed in a 96-well fluorescence microplate reader (HTS 7000, Perkin Elmer). As shown in **FIG. 20**, approximately 80 % of the clones in this library are inactive or less active mutants, as compared to wild type $P450_{cam}$. A percentage (about 20%) of the randomly selected clones exhibited improved naphthalene hydroxylation activity. This result is similar to that obtained using fluorescence image analysis (**FIG 19**). However, the image analysis is much faster and less expensive (estimated analysis time: approximately 3-5 seconds for analysis of 20,000 colonies).

D. Kinetic characterization of P450cam mutants

Five positive $P450_{cam}$ variants (designated M7-4H, M7-6H, M7-8H, M7-9H, and M7-2R) were selected from among about 200,000 colonies (Q-bot : 9 plates) which were screened by fluorescence image analysis. Three clones with fluorescence values near the threshold (wild type activity) were also selected for comparison (M7-1, M7-2, M7-3). These clones were grown and analyzed in a 96-well plate format for activity towards three different substrates, naphthalene, 3-phenylpropionate and coumarin. One clone, M7-2R, proved to be a false positive and was not analyzed further. Results of the kinetic analysis are summarized in TABLE 2.

TABLE 2

Relative rates for $P450_{cam}$ variants towards 3-phenylpropionate, coumarin and naphthalene, as measured by generation of fluorescence per time in a 96-well plate assay using whole cells.

Substrate	WT	Positive Variants				Controls		
		M7-4H	M7-6H	M7-8H	M7-9H	M7-1	M7-2	M7-3
3-phenyl-propionate	13.8	42.8	43.6	35.4	33.0	7.8	9.0	16.2
coumarin	8.2	11.5	14.1	12.8	9.3	2.6	1.2	3.1
naphthalene	9.2	84.1	86.7	53.1	82.9	5.4	6.7	11.4

For naphthalene hydroxylation, variant M7-6H showed 9.4-fold increased activity over the wild type. Four of the P450 positives showed highly improved activity towards naphthalene and also towards the 3-phenylpropionate. In another series of experiments,

M7-6H showed an 11-fold increase compared to wild-type on naphthalene, and M7-4H and M4-8H showed at least a 5 to 8 fold increase in activity. These three clones also had increased activity on 3-phenylpropionate, with M7-6H showing a 3.2 fold increase. The activity towards coumarin, as measured in this assay, was only slightly increased.

For the microtitre plate assay, the cells (grown in 4 ml TB/amp (100 ug/ml) media) were centrifuged for 10 min at 4°C. After the supernatant solution was removed, the harvested cells were carefully resuspended in 1 ml buffer solution (dibasic phosphate, 100 mM, pH 9.0). Then, 20 µl aliquots were placed into a Nunc fluorescence microplate. The total 180 µl reaction mixture was made up of 100 µl dibasic sodium phosphate buffer (100 mM, pH9.0), 20 µl ethanol, 10 µl substrate stock (4.5 mM coumarin in 10% ethanol, or 2 mM 3-phenylpropionate in 10 % ethanol, 2 mM naphthalene in pure ethanol), and 10 µl hydrogen peroxide stock solution (50 mM H_2O_2 stock). The other reaction conditions are those described in EXAMPLE 2. The fluorescence was measured as a function of time, and the relative rates presented in Table 2 are the slopes of that measurement (RFU/min).

E. Sequence characterization of P450cam mutants

Sequence analysis of three $P450_{cam}$ mutant clones of the invention, M7-4H, M7-6H, and M7-8H, revealed a mutation at position 331 of the amino acid sequence of **FIG. 3B**, in which glutamic acid (Glu or E) has been changed to lysine (Lys or K). In mutant M7-4H this was the only mutation **[SEQ ID NO: 11]**. Mutant M7-6H was found to have a second mutation at position 280 of the amino acid sequence of **FIG. 3B**, in which arginine (Arg or R) is changed to leucine (Leu or L) **[SEQ ID NO: 12]**. Mutant M7-8H was found to have a second mutation at position 242 of the amino acid sequence of **FIG. 3B**, in which cysteine (Cys or C) is changed to phenylalanine (Phe or F) **[SEQ ID NO: 13]**.

F. Regiospecific P450 Enzymes

Reaction products of the oxygenation reaction catalyzed by P450 enzyme were reacted in the presence of HRP and hydrogen peroxide. *In vitro* HRP-catalyzed polymerization of different naphthol isomers (alpha and beta) and different dihydroxylated naphthalenes (1,5-dihydroxy-, 2,3 dihydroxy- and 2,7-dihydroxy-) generated a variety of fluorescent products, ranging from dark blue (430-460 nm), blue-green (495 nm), yellow (580 nm) to orange-red (620 nm) fluorescence. A combination of 1- or 2-naphthol and 2,7-dihydroxy naphthalene produces a red fluorescent product (620 nm), while mixing 1,5-

dihydroxy naphthalene with 2,7-dihydroxy naphthalene or 2-naphthol produces pink and yellow fluorescence, respectively. The emission spectra depend on the relative molar ratios of the reactants.

Bacteria expressing wild-type $P450_{cam}$ generate only blue fluorescence (460nm), corresponding to the conversion of naphthalene to 1- or 2-naphthol (and coupling by HRP). Bacteria expressing the $P450_{cam}$ mutants, in contrast, generate a palette of colors, shown in Table 3 below, that reflect the altered regiospecificities of the $P450_{cam}$-catalyzed hydroxylations. Thus, the screen according to the invention is sensitive to regiospecificity of hydroxylation as well as overall monooxygenase activity.

TABLE 3

Color reactions produced by Mutant Clones

	1	2	3	4	5	6	7	8	9	10	11	12
A	WK-BL	BL	BR-BL	PK	YL	YL	YL	BR-BL	BR-BL	BR-BL	YL	BR-BL
B	WK-BL	BL	BR-BL	BR-BL	YL	BR-BL	BR-BL	BR-BL	BL	BR-BL	BR-BL	BL
C	WK-BL	BL	PK	BR-BL	BL	BL	YL	BR-BL	RD	BR-BL	ST-YL	
D	WK-BL	BL	BR-BL	BL	BR-BL	BR-BL	BR-BL	BR-BL	BR-BL	YL	BL	BL
E	WK-BL	BL	BR-BL	BR-BL	PK	BR-BL	BR-BL	BL	BR-BL	BR-BL	BR-BL	BL

*legends : WK : weak, ST: strong, BR; bright, BL: blue, YL; yellow, RD; red

Rows A-E of Column 1 correspond to the control strain, *E. coli* BL21(DE3). Column 2 (Rows A-E) corresponds to the control strain expressing native $P450_{cam}$. The remaining 10 columns show 50 different variants selected by fluorescence image scanning on naphthalene as substrate. Naphthalene hydroxylation activities were measured in 200 µL reactions in the 96 well plate. Cells grown in 50 ml flasks were harvested by centrifugation (Beckman CS SR) at 3350 rpm and resuspended in 1 mL of 0.1 M sodium dibasic buffer (pH 9.0). A 50 µL aliquot of this solution was added to the same buffer mixtures (total of 200 µL) containing 25% ethanol, naphthalene (6 mM) and hydrogen peroxide (10 mM). Fluorescence was measured using a 96 well microfluorimeter (Perkin Elmer HTS 7000).

The screen is also selective for one of the hydroxylated isomers of 3-phenylpropionate (3-PPA). Although an oxygenase can potentially hydroxylate different positions on the aromatic backbone of 3-PPA, the product hydroxylated at the 4-position, 3-(4-hyroxyphenyl)propionate, generates strong blue fluorescence (emission at 465 nm, 350 nm excitation) when coupled with HRP. In contrast, HRP does not generate any detectable fluorescence with 3-(2-hydroxyphenyl) propionate as the substrate in an *in vitro* assay.

The genes encoding these and other improved P450 variants can be recombined by DNA shuffling methods or they can be further mutated in additional cycles of directed evolution or error prone PCR in order to generate further improved enzymes. P450s with improved thermostability, for example, can be obtained by measuring residual activity after incubation at elevated temperature.

EXAMPLE 9

Expression of Horseradish Peroxidase in *E. coli* and Yeast

A. Cloning of HRP

The HRP gene was cloned from the plasmid pBBG10 (British Biotechnologies, Ltd., Oxford, UK) by PCR techniques to introduce an *Msc* I site at the start codon and an *EcoR* I site immediately downstream from the stop codon. This plasmid contains the synthetic horseradish peroxidase (HRP) gene described in Smith et al. (26), whose DNA sequence is based on a published amino acid sequence for the HRP protein (38). pBBG10 was made by inserting the HRP sequence between the HinDIII and EcoR1 sites of the polylinker in the well-known plasmid PUC19. The PCR product obtained from this plasmid was digested with Msc I and EcoR I and ligated into similarly digested pET-22b(+) (purchased from Novagen) to yield pETpelBHRP. A map of this expression vector shown in **FIG 21.** In this construct, the HRP gene was placed under the control of the T7 promoter and is fused in-frame to the pelB signal sequence (*See* **[SEQ ID NO: 14]** and **FIG. 22**), which theoretically directs transport of proteins into the periplasmic space, that is, for delivery outside the cell cytoplasm (25). The ligation product was transformed into *E. coli* strain BL21(DE3) for expression of the protein in cells both with and without induction by 1 mM IPTG.

In the cells that were induced with IPTG, no peroxidase activity above background was detected, for BL21(DE3) cells or pET-22b(+)-harboring BL21(DE3) cells, even though the level of HRP polypeptides accounted for over 20% of total cellular proteins. This was consistent with previous observations (26, 27, 28).

In the cells that were not induced with IPTG, clones were discovered that showed weak but measurable activity against azino-di-(ethylbenzthiazoline sulfonate (ABTS).

The T7 promoter in the pET-22b(+) vector is known to be leaky (29), and in theory it is therefore possible that some of the HRP polypeptide chains produced at this basal level were able to fold into the native form. Conversely, addition of IPTG leads to high-level HRP synthesis, which instead favors aggregation of chains and prevents their proper folding. Subsequently, random mutagenesis and screening were used to identify mutations that might lead to higher expression of HRP activity.

B. Random library generation and screening

One of the HRP clones that showed detectable peroxidase activity was used in the first generation of error-prone PCR mutagenesis. The random libraries were generated by a modification of the previously described error-prone PCR protocol (15, 30), in which 0.15 mM of $MnCl_2$ was used instead of 0.5 mM $MnCl_2$. This protocol incorporates both manganese ions and unbalanced nucleotides, and has been shown to generate both transitions and transversions and therefore a broader spectrum of amino acid changes (31).

Briefly, the PCR reaction solution contained 20 fmoles template, 30 pmoles of each of two primers, 7 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 0.15 mM $MnCl_2$, and 5 unit of Taq polymerase in a 100 µl volume. PCR reactions were performed in a MJ PTC-200 cycler (MJ Research, MA) for 30 cycles with the following parameters: 94 C for 1 min, 50 C for 1 min, and 72°C for 1 min. The primers used were:

5'-TTATTGCTCAGCGGTGGCAGCAGC [SEQ ID NO: 18], and

5'-AAGCGCTCATGAGCCCGAAGTGGC [SEQ ID NO: 19].

The PCR products were purified with a Promega Wizard PCR kit, and digested with Nde I and Hind III. The digestion products were subjected to gel-purification with a QIAEX II gel extraction kit, and the HRP fragments were ligated back into the similarly digested and gel-purified pET-22b(+) vector. Ligation mixtures were transformed in the BL21(DE3) cells by electroporation with a Gene Pulser II (Bio-Rad).

The PCR products were ligated back into the pET-22b(+) vector which was transformed into the BL21(DE3) cells by electroporation. Cell growth and expression was carried out in either 96-well or 384-well microplates in LB medium at 30°C. Peroxidase activity tests were performed with H_2O_2 and ABTS (32).

For each generation, typically 12,000-15,000 colonies were picked and screened in 96-well plates. This number represents an exhaustive search of all accessible single mutants, with a probability of 95% for any mutant to be sampled at least once (33). Colonies were either picked manually, or using an automated colony picker at Caltech, Q-bot (Genetix, UK).

Of the 12,000 colonies that were screened in the first generation (no IPTG added), a clone designated HRP1A6 showed 10-14 fold higher peroxidase activity than the parent clone. This clone also showed markedly decreased activity when as little as 5 μM of IPTG was added. Sigma reports that 1 mg of highly purified HRP from horseradish has a total activity of 1,000 units, as determined by the ABTS assay. Other workers reported similar results (26). Based on this data, the concentration of active HRP was estimated to be about 100 ug/L. HRP1A6 shows a total activity of greater than 100 units/L. This compares favorably with the yield obtained from refolding of aggregated HRP chains in vitro (26). This level of expression for the HRP1A6 clones also similar to that for bovine pancreatic trypsin inhibitor (BPTI) in *E. coli* (34), an unglycosylated protein with three disulfide bonds. Once again, greater than 95% of the HRP activity was found in the LB culture medium as judged by the ABTS activity.

The HRP1A6 clone remained stable for up to a week at 4°C. IPTG was omitted in all HRP expression experiments, unless otherwise specified. Peroxidase activity tests for HRP were performed with a classical peroxidase assay, ABTS and hydrogen peroxide (26). Fifteen μl of cell suspension was mixed with 140 μl of ABTS/H_2O_2 (2.9 mM ABTS, 0.5 mM H_2O_2 , pH 4.5) in microplates, and the activity was determined with a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) at 25°C. A unit of HRP is defined as the amount of enzyme that oxidizes 1 μmole of ABTS per min at the assay conditions.

HRP1A6 demonstrated higher expression and/or peroxidase activity than wild-type HRP. Sequencing of the HRP1A6 gene revealed that its amino acid sequence is identical to that of wild-type HRP. This indicates that the HRP1A6 gene contains a mutation outside of the protein encoding region that results in an enhanced peroxidase activity of this clone. A map of the plasmid pETpelBHRP1A6 containing the HRP1A6 gene is shown in **FIG. 24.**

C. *Functional Expression of HRP in Yeast*

The native HRP protein contains four disulfide bonds, and *E. coli* has only a limited capability to support disulfide formation. In theory, these well-conserved disulfides in HRP

(and other plant peroxidases) are likely to be important for the structural integrity of the protein, and may not be replaceable by mutations elsewhere. Yeast has a much greater ability to support the formation of disulfide bonds. Thus, yeast can be used as suitable expression host, in place of *E. coli*, particularly if it s desired to relieve the apparent limitation on the folding of HRP imposed by any constraints on disulfide formation in *E. coli*. For example, *S. cerevisiae* can be used as a host for the expression of mutant HRP genes and proteins.

The recombinant wild-type HRP gene HRP1A6 was cloned into the secretion vector pYEX-S1 obtained from Clontech (Palo Alto, CA) (19), yielding pYEXS1-HRP (**FIG. 25**). This vector utilizes the constitutive phosphoglycerate kinase promoter and a secretion signal peptide from *Kluveromyces lactis* . The plasmid was first propagated in *E. coli*, and then transformed into *S. cerevisiae strain* BJ5464, obtained from the Yeast Genetic Stock Center (YGSC), University of California, Berkeley using the LiAc method as described (20). BJ5464 is protease deficient, and has been found to be generally suitable for secretion.

A first generation of error-prone PCR of HRP in yeast was performed. Among the first 7,400 mutants screened, four variants showed 400% higher activity than HRP1A6 in yeast.

EXAMPLE 10

Screening for Other Catalysts and Optimizing Reaction Conditions

Empirical approaches are the only proven successful approaches to the development of novel catalysts. However, empirical approaches are often slow, costly and labor-intensive. Parallel investigation of a large number of catalyst candidates can significantly reduce the time, cost and labor associated with catalyst discovery. In addition to enzymatic catalysts, the methods of this invention can be applied to screen chemical libraries for oxidation catalysts.

In addition to catalyst discovery, it is also important to optimize reaction conditions for any given catalyst. This requires the simultaneous optimization of a number of parameters, each of which can have a significant effect on catalyst performance. Important parameters include choice of solvent, reactant profile, presence of other compounds or contaminants in the reaction mixture, temperature, pressure etc. Given the large number of potential variables, optimization is also preferably done in parallel tests, in which dozens or

even thousands of conditions are tested. Once an oxidation catalyst is in hand, the invention can be used to rapidly evaluate or optimize conditions for that catalyst.

The invention can be used with single catalysts (*e.g.* arrayed in individual wells of a microtiter plate) or it can be used with various pooling strategies in which multiple candidate catalysts are assayed simultaneously. If a particular set of catalysts shows reactivity in a given reaction, the members of that set can be assayed individually to discover the catalyst of interest.

A. Combinatorial Approaches to Catalyst Design and Discovery.

The invention can be used to screen libraries of non-enzyme catalysts for their ability to oxygenate (*e.g.* hydroxylate) substrates, such as aromatic substrates. Catalysts identified in this way can in turn be used as "leads" for the discovery of catalysts that hydroxylate other substrates, catalyze other oxygen insertion reactions, or which have more activity or stability, or which can function under different conditions. For example, the techniques of combinatorial chemistry can be used to generate additional libraries of compounds for testing, once a lead compound is identified (43, 48, 49).

To use the invention for this application, the screening reaction with the coupling enzyme would generally be performed after the oxygenation reaction has completed. If necessary or appropriate, the reaction conditions can be adjusted after the oxygenation reaction, so as to promote the coupling reaction. That is, conditions that are compatible with maintaining the activity of the coupling enzyme must be provided. Alternatively, the oxygenated products could be extracted into a solvent (e.g. dichloromethane or a solvent in which the coupling enzyme, such as HRP, is known to function). HRP, a preferred coupling enzyme, is known to function as a coupling enzyme in aqueous buffer and also in various organic solvents (including hexane, acetonitrile, t-butanol and others) and functions over a temperature range of approximately 4 to 65 °C, with best performance around 20-50 °C. The coupling reaction conditions can be readily tested to determine that they support the activity of the coupling enzyme. The coupling reaction conditions are also preferably chosen to minimize dilution of the oxygenated product.

This embodiment also allows measurement of an "end point" of the oxygenation reaction. For example, the oxygenation reaction would be allowed to proceed for a given amount of time. At this point, the conditions are changed to allow the coupling reaction (and coupling enzyme and oxygen donor would be added). The generation of colored or

fluorescent products (or absorption of UV light, chemiluminescence, etc.) indicates the total concentration of the oxygenated product made during that time. If the oxygenation catalyst functions under conditions that are also compatible with the coupling enzyme, both reactions be done simultaneously or contemporaneously (oxygenation and coupling).

B. Optimizing Reaction Conditions.

The invention can also be used to optimize reaction conditions for any given catalyst.

The hydroxylation of aromatic compounds can be is difficult. Results can be poor because introduction of a hydroxyl group activates the ring for further reaction and oxidation. Furthermore, the reaction conditions are often harsh and potentially explosive (45). Thus, evaluating and optimizing reaction conditions for a given catalyst can be beneficial.

There are various non-enzyme catalysts that are known to catalyze aromatic hydroxylations, similar to monooxygenase and dioxygenase enzymes. DeHaan et al. (46) describe hydroxylation of various aromatics in high yield, using a bis(trimethylsilyl)peroxide/triflic acid system. The product was extracted into an organic solvent (dichloromethane) for analysis. The present invention can be used to determine the progress of the reaction by adding HRP (or other suitable coupling enzyme) and peroxide. Alternatively, a solvent that both extracts the product and supports the activity of the HRP can be used.

As another example, a large class of catalysts that can perform hydroxylations are the substituted porphyrins, which have been characterized as non-enzyme "mimics" of P450 enzymes (47). This invention can be used to screen combinatorial libraries of porphyrin-based catalysts for hydroxylation of aromatics under a variety of conditions. It can also be used to screen libraries of di-iron compounds that mimic di-iron oxygenases (51).

Having thus described exemplary embodiments of the invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the invention. For example, it will be understood by practitioners that the steps of any method of the invention can generally be performed in any order, including simultaneously or contemporaneously, unless a particular order is expressly required, or is necessarily inherent or implicit in order to practice the invention. Accordingly, the invention is not limited to any specific embodiments or illustrations herein. The invention is defined according to the appended claims, and is limited only according to the claims.

BIBLIOGRAPHY

1. Faber, K. Biotransformations in Organic Chemistry, Springer-Verlad, Berlin, p. 214, 217 (1997)

2. Cook, D.L. and Atkins, W.M. *Biochemistry*, **36**, 10801 (1997).

3. Short, J. *Nature Biotechnol.* **15**, 1322 (1997).

4. Sheldon, R.A. Catalysis: the key to waste minimization. *J. Chem. Tech. Biotechnol.* **68**, 381 (1997).

5. Gonzalez, F.J. and Nebert, D.W., Evolution of the P450-gene superfamily - animal plant warfare, molecular drive and human genetic differences in drug oxidation. *Trends Genet.* **6**, 182-186 (1990).(1975).

6. Guengerich, F.P. in Cytochrome P450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano, P.R., E.d.) pp. 473-536, Plenum Press, New York (1995).

7. England, P.A., Harford-Cross, C.F., Stevenson, J.-A., Rouch, D.A., and Wong, L.-L., *FEBS Lett.* **424**, 271-274 (1998).

8. Lipscomb, J.D., Sligar, S.G., Namtvedt, M.J. Gunsalus, I.C. *J. Biol. Chem.*, **251**, 1116 (1976).

9. Blake II, R. C. and Coon, M. *J. Biol. Chem.*, **255**, 4100 (1980).

10. van Deurzen, M.P.J., Van Rantwijk, F., Sheldon, R.A. *Tetrahedron*, **53**, 13183 (1997).

11. Nordblom, G.D., White, R.E., and Coon, M. *J. Arch. Biochem. Biophys.*, **175**, 524 (1976).

12. Rahimtula, A.D. and P.J. O'Brien *Biochem. Biophys. Res. Commun.* **60**, 440 (1974).

13. U.S. Patent No. 5,741,691 and U.S. Patent No. 5,811,238.

14. Mueller, E. J., Loida, P. J., and Sligar, S. G., Twenty-five Years of $P450_{cam}$ Research,

in *Cytochrome P450 : Structure, Mechanism, and Biochemistry* (2nd ed. Montellano, P. R. O. de), Plenum Press, NY, pp83-124(1995).

15. Cadwell, R. C. and Joyce, G. F., Randomization of Genes by PCR Mutagenesis, in: *PCR Methods & Applications*, Cold Spring Harbor Laboratory Press, NY, pp28-33 (1992).

16. U.S. Patent No. 5,605,793

17. PCT Application No. PCT/US98/05956

18. D. R. Thatcher, A. Hitchcock, in *Mechanisms of Protein Folding* R. H. Pain, Ed. (IRL Press, Oxford, 1994) pp. 229-261.

19. C. B. Anfinsen, *Science* **181**, 223-230 (1973).

20. C. H. Schein, *Bio/Technology* **8**, 308-317 (1990).

21. A. Mitraki, J. King, *FEBS Lett.* **307**, 20-25 (1992).

22. J. X. Zhang, D. P. Goldenberg, *Biochemistry* **32**, 14075-14080 (1993).

23. R. Wetzel, L. P. Perry, C. Veilleux, *Bio/Technology* 9, 731-737 (1991).

24. A. Crameri, E. A. Whiteborn, E. Tate, W. P. C. Stemmer, *Nature Biotechnol.* **14**, 315-319 (1996).

25. S. P. Lei, H. C. Lin, S. S. Wang, J. Callaway, G. Wilcox, *J. Bacteriol.* **169**, 4379-4383 (1987).

26. A. T. Smith, et al., *J. Biol. Chem.* **265**, 13335-13343 (1990).

27. A. M. Egorov, et al., *Ann. N. Y. Acad. Sci.* , 35-40 (1991).

28. S. A. Ortlepp, D. Pollard-Knight, D. J. Chiswell, *J. Biotechnol.* **11**, 353-364 (1989)

29. F. W. Studier, A. H. Rosenberg, J. J. Dunn, J. W. Dubendorff, *Meth. Enzymol.* **185**, 60-89 (1990).

30. S. Shafikhani, R. A. Siegel, E. Ferrari, V. Schellenberger, *Biotechniques* **23**, 304-310 (1997).

31. K. Sirotkin, *J. Theor. Biol.* **123**, 261-279 (1986).

32. J. S. Shindler, R. E. Childs, W. G. Bardsley, *Eur. J. Biochem.* **65**, 325-331 (1976)

33. J. Carbon, L. Clarke, C. Ilgen, B. Ratzkin, in *Recombinant Molecules: Impact on Science and Society* R. F. J. Beers, E. G. Bassett, Eds. (Raven Press, New York, 1977).

34. M. Ostermeier, K. Desutter, G. Georgiou, *J. Biol. Chem.* **271**, 10616-10622 (1996)

35. R. Parekh, K. Forrester, D. Wittrup, *Protein Expres. Purif.* **6**, 537-545 (1995).

36. R. D. Gietz, R. H. Schiestl, A. Willems, R. A. Woods, *Yeast* **11**, 355-360 (1995)

37. Riggs, P., in Ausubel, F. M., et al. (eds), Current Protocols in Molecular Biology (1992) Greene Associates/Wiley Interscience, New York.

38. K. G. Welinder, *Eur. J. Biochem.* **96**, 483-502 (1979).

39. D. B. Goodin, M. G. Davidson, J. A. Roe, A. G. Mauk, and M. Smith, Biochemistry 30, 4953-4962 (1991)

40. M. M. Fitzgerald, M. J. Churchill, D. E. McRee, and D. B. Goodin, Biochemistry, 33, 3807-3818 (1994).

41. Zylstra, G. J. and Gibson, D. T. (1989) J. Bacteriol. 264, 14940-14946.

42. Miura, Y. and Fulco, A.J. *Biochim. Biophys. Acta*, **388**, 305 (1975).

43. Borchardt, J.K., Combinatorial Chemistry: Not just for pharmaceuticals. *Today's Chem. at Work*, November 1998, pp. 36-39.

44. Setti, L. et al., Horseradish peroxidase-catalyzed oxidative coupling of 3-methyl 2-benzothiazolinone hydrazone and methoxyphenols. *Enz. & Mocrob. Tech.*, 22:656-661 (1998).

45. G. A. Olah & T. D. Ernst, Oxyfunctionalization of Hydrocarbons. 14. Electrophilic Hydroxylation of Aromatics with Bis(trimethylsilyl)peroxide/Triflic Acid, *J. Org. Chem.* 54: 1204-1206 (1989).

46. DeHaan et al., [CITE]

47. J.T. Groves & Y.-Z. Han, Models and Mechanisms of Cytochrome P450 Action, in Cytochrome P450, 2nd Edition, Ed. P. R. Ortiz de Montellano, Plenum, NY pp3-48.

48. A. H. Hoveyda, Catalyst discovery through combinatorial chemistry, *Chemistry & Biology* 5:R187-R191 (1998).

49. Stuart Borman, Combinatorial Catalysts, *Chemical & Engineering News*, Nov. 4, 1996 p. 37-39.

50. Handelsman, J. et al., Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products, *Chem. & Biol.*, 5:R245-249 (1998).

51. Menage, S. et al., O_2 activation and aromatic hydroxylation performed by diiron complexes, *J. Am. Chem. Soc.*, 120, 133370-13382 (1998).

52. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual;* Cold Spring Harbor Laboratory: New York (1989).

53. Stemmer, W. P. C. *et al.*, *Biotechniques* **14**, 256 (1992).

WE CLAIM:

1. A method for detecting an oxidation enzyme comprising the steps of:
   a) providing an organic substrate and an oxygen donor;
   b) supplying a test enzyme comprising a protein-containing material selected for evaluation of its ability to influence the formation of an oxygen-containing product from the organic substrate and the oxygen donor;
   c) introducing the test enzyme to the organic substrate and the oxygen donor;
   d) furnishing a coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product; and
   e) testing for the detectable composition, wherein the presence of the detectable composition indicates that the test enzyme is an oxidation enzyme.

2. The method of claim 1, wherein the test enzyme is introduced to the organic substrate and the oxygen donor under reaction conditions which are selected to evaluate the ability of the test enzyme to mediate the addition of oxygen to the substrate.

3. The method of claim 2, wherein the reaction conditions are varied.

4. The method of claim 2, wherein the coupling enzyme is furnished under coupling conditions selected to promote formation of a polymeric oxygenated composition comprising two or more of the same oxygen-containing products joined to each other.

5. The method of claim 1, wherein the organic substrate is an aromatic compound.

6. The method of claim 1, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

7. The method of claim 1, wherein the oxygen donor is molecular oxygen.

8. The method of claim 1, wherein the oxygen donor is a peroxide.

9. The method of claim 8, wherein the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide.

10. The method of claim 1, wherein the coupling enzyme is used to bring together molecules of the oxygen-containing product to form a polymeric composition that is detectable using at least one of ultraviolet light, a color change, fluorescence and luminescence.

11. The method of claim 1, wherein the coupling enzyme is a peroxidase enzyme.

12. The method of claim 6, wherein the coupling enzyme is a peroxidase enzyme.

13. The method of claim 8, wherein the coupling enzyme is a peroxidase enzyme.

14. The method of claim 1, wherein the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and peroxidase Novozyme® 502.

15. The method of claim 1, wherein the coupling enzyme is a laccase enzyme.

16. The method of claim 1, wherein the organic substrate is an aromatic compound, the oxygen donor is a peroxide and the coupling enzyme is a peroxidase enzyme.

17. The method of claim 16, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

18. The method of claim 16, wherein the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, an peroxidase Novozyme® 502.

19. The method of claim 1, further comprising the step of providing a chemiluminescent agent.

20. A method for detecting an oxygenase enzyme comprising the steps of:

a) providing an aromatic organic substrate and a peroxide oxygen donor;

b) supplying a test enzyme comprising a protein-containing material selected for evaluation of its ability to influence the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

c) introducing the test enzyme to the organic substrate and the oxygen donor;

d) furnishing a peroxide coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product; and

e) testing for the detectable composition by evaluating at least one of ultraviolet light, a color change, fluorescence, and luminescence, wherein the presence of the detectable composition indicates that the test enzyme is an oxygenase enzyme.

21. The method of claim 20, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

22. The method of claim 20, wherein the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide.

23. The method of claim 20, wherein the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and peroxidase Novozyme® 502.

24. The method of claim 20, further comprising the step of providing a chemiluminescent agent.

25. The method of claim 23, further comprising the step of providing a chemiluminescent agent.

26. The method of claim 20, wherein the chemiluminescent agent is luminol.

27. The method of claim 23, wherein the chemiluminescent agent is luminol.

28. The method of claim 20, wherein the oxygenase enzyme is one of a dioxygenase enzyme and a monooxygenase enzyme.

29. The method of claim 20, wherein the reaction catalyzed by the oxygenase enzyme is one of a hydroxylation reaction, an epoxidation reaction, and a sulfoxidation reaction.

30. The method of claim 1, wherein the oxygenase enzyme is selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

31. The method of claim 20, wherein the oxygenase enzyme is selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

32. The method of claim 20, wherein the organic substrate is naphthalene.

33. The method of claim 20, wherein the oxygen donor is hydrogen peroxide.

34. The method of claim 20, wherein the coupling enzyme is horseradish peroxidase.

35. The method of claim 1, wherein the introducing step comprises introducing the organic substrate and oxygen donor to host cells that express at least one of a test enzyme and a coupling enzyme.

36. The method of claim 20, wherein the introducing step comprises introducing the organic substrate and oxygen donor to host cells that express at least one of a test enzyme and a coupling enzyme.

37. The method of claim 1, wherein the test enzyme is a variant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

38. The method of claim 20, wherein the test enzyme is a variant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

39. The method of claim 35, wherein the host cells are bacterial cells.

40. The method of claim 36, wherein the host cells are bacterial cells.

41. The method of claim 39, wherein the host cells are E. coli cells.

42. The method of claim 40, wherein the host cells are E. coli cells.

43. The method of claim 45, wherein the host cells are yeast cells.

44. The method of claim 36, wherein the host cells are yeast cells.

45. The method of claim 43, wherein the host cells are S. cerevisiae cells.

46. The method of claim 44, wherein the host cells are S. cerevisiae cells.

47. The method of claim 20, wherein:

a) the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin;

b) the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide; and

c) the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and peroxidase Novozyme® 502.

48. The method of claim 47, wherein the test enzyme is a variant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

49. The method of claim 48, further comprising the step of providing a chemiluminescent agent.

50. The method of claim 1, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the absence of at least one coenzyme or ancillary protein.

51. The method of claim 20, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the absence of at least one of its coenzymes or ancillary proteins.

52. The method of claim 47, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the absence of at least one of its coenzymes or ancillary proteins.

53. The method of claim 50, wherein at least one coenzyme is selected from the group consisting of nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH).

54. The method of claim 50, wherein at least one ancillary protein is selected from the group consisting of putidaredoxin and putidaredoxin reductase.

55. The method of claim 51, wherein at least one coenzyme is selected from the group consisting of nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH).

56. The method of claim 51, wherein at least one ancillary protein is selected from the group consisting of putidaredoxin and putidaredoxin reductase.

57. The method of claim 1, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the presence of one or more cofactors.

58. A method for detecting an oxygenase enzyme comprising the steps of:

a) providing an aromatic organic substrate and a peroxide oxygen donor;

b) supplying a test enzyme comprising a protein-containing material selected for evaluation of its ability to influence the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

c) introducing the test enzyme to the organic substrate and the oxygen donor in the absence of at least one of its coenzymes or ancillary proteins;

d) furnishing a peroxide coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product; and

e) testing for the detectable composition by evaluating at least one of ultraviolet light, a color change, fluorescence, and luminescence, wherein the presence of the detectable composition indicates that the test enzyme is an oxygenase enzyme.

59. The method of claim 63, wherein at least one coenzyme is selected from the group consisting of nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH).

60. The method of claim 58, wherein at least one ancillary protein is selected from the group consisting of putidaredoxin and putidaredoxin reductase.

61. The method of claim 58, wherein the organic substrate, oxygen donor, and test enzyme are introduced in the presence of one or more cofactors.

62. The method of claim 61, wherein at least one cofactor is selected from the group consisting of thiamine (vitamin B1), ferrous chloride (FeCl2) and delta-aminolevulinic acid (ALA).

63. The method of claim 58, wherein the test enzyme is a variant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

64. The method of claim 38, wherein at least one coenzyme is selected from the group consisting of nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH), or at least one ancillary protein is selected from the group consisting of putidaredoxin and putidaredoxin reductase.

65. A method for evaluating a test enzyme for its ability to mediate production of an oxygenated product when introduced to an organic substrate in the presence of an oxygen donor, comprising the steps of:

a) providing a host cell which is capable of expressing a test enzyme;

b) furnishing a vector which encodes the test enzyme;

c) inserting the vector into the host cell to provide a transformed host cell that expresses the test enzyme;

d) supplying an oxygen donor;

e) introducing an organic substrate to provide a reaction with the oxygen donor in the presence of the test enzyme to produce an oxygenated product;

f) reacting the oxygenated product in the presence of a coupling enzyme to form a polymeric oxygenated product; and

g) detecting the polymeric oxygenated product by testing for at least one of fluorescence, luminescence, ultraviolet light, and a color change.

66. The method of claim 65 wherein the host cell further expresses the coupling enzyme.

67. The method of claim 65 comprising the further step of introducing a second vector which expresses the coupling enzyme into the host cell.

68. The method of claim 65 comprising the further step of reacting the polymeric oxygenated product with a chemiluminescent agent to produce a chemiluminescent compound, and the detecting step comprises evaluating the luminescence of the chemiluminescent compound.

69. The method of claim 65, wherein the test enzyme is a mutant oxygenase enzyme.

70. The method of claim 69, wherein the mutant oxygenase enzyme is mutant of an enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase enzymes.

71. The method of claim 65, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

72. The method of claim 65, wherein the oxygen donor is a peroxide.

73. The method of claim 65, wherein the coupling enzyme is a peroxidase.

74. The method of claim 69, wherein the mutant oxygenase enzyme is a mutant of a P450 enzyme.

75. The method of claim 74, wherein the oxygen donor is a peroxide.

76. The method of claim 74, wherein the coupling enzyme is a peroxidase.

77. The method of claim 74, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin, the oxygen donor is a peroxide, and the coupling enzyme is a peroxidase.

78. The method of claim 75, wherein the coupling enzyme is horseradish peroxidase.

79. The method of claim 74, wherein the P450 enzyme is a P450cam enzyme.

80. The method of claim 1, wherein the test enzyme is a mutant enzyme obtained by at least one of random mutagenesis, site-specific mutagenesis, and DNA shuffling.

81. The method of claim 20, wherein the test enzyme is a mutant enzyme obtained by at least one of random mutagenesis, site-specific mutagenesis, and DNA shuffling.

82. The method of claim 58, wherein the test enzyme is a mutant enzyme obtained by at least one of random mutagenesis, site-specific mutagenesis, and DNA shuffling.

83. The method of claim 65, wherein the test enzyme is a mutant enzyme obtained by at least one of random mutagenesis, site-specific mutagenesis, and DNA shuffling.

84. The method of claim 65, wherein the vector comprises a variant of the P450cam nucleotide sequence of FIG. 3A [SEQ ID NO: 1].

85. The method of claim 65, wherein the vector encodes a mutation of the P450cam amino acid sequence of FIG. 3B [SEQ ID NO: 2].

86. The method of claim 66, wherein the second vector comprises the horseradish peroxidase nucleotide sequence of FIG. 23 [SEQ ID NO: 16].

87. The method of claim 66, wherein the second vector encodes the horseradish peroxidase amino acid sequence of FIG. 23 [SEQ ID NO: 17].

88. A method of screening for oxygenase enzymes comprising the steps of:

a) providing host cells;

b) furnishing a plurality of vectors each of which encodes a test enzyme;

c) inserting each vector into one or more host cells to provide corresponding transformed cells that each express a corresponding test enzyme;

d) introducing each test enzyme to an oxygen donor and an organic substrate under conditions which provide for a reaction between the substrate and donor to produce an oxygenated product;

e) reacting the oxygenated product in the presence of a coupling enzyme to form a polymeric oxygenated product; and

f) detecting the polymeric oxygenated product by testing for the presence or degree of at least one indicator selected from the group consisting of fluorescence, luminescence, ultraviolet light, and a color change, wherein detection of the polymeric oxygenated product indicates that a corresponding test enzyme is an oxygenase.

89. The method of claim 88, wherein a plurality of the oxygenase enzymes are compared to each other by evaluating the corresponding degrees of detected indicator.

90. The method of claim 88, further comprising the step of reacting the polymeric oxygenated product with a chemiluminescent agent to form a chemiluminescent composition, and wherein the detecting step comprises testing for luminescence of the chemiluminescent composition.

91. The method of claim 88, wherein each vector encodes a test enzyme that is a variant of an oxygenase enzyme.

92. The method of claim 88, wherein each test enzyme is a variant or an oxygenase enzyme selected from the group consisting of chloroperoxidase, cytochrome P450, methane monooxygenase, toluene monooxygenase, toluene dioxygenase, biphenyl dioxygenase and naphthalene dioxygenase.

93. The method of claim 88, wherein each vector encodes a test enzyme that is a variant enzyme obtained by at least one of random mutagenesis, specific mutagenesis, directed evolution, DNA shuffling, and error-prone polymerase chain reaction.

94. The method of claim 88, wherein the oxygen donor is a peroxide.

95. The method of claim 92, wherein the oxygen donor is a peroxide, the coupling enzyme is a peroxidase, and the organic substrate is a aromatic compound selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

96. The method of claim 58, wherein the detecting step includes image analysis of detected ultraviolet light, color change, fluorescence or luminescence.

97. The method of claim 65, wherein the detecting step includes image analysis of detected ultraviolet light, color change, fluorescence or luminescence.

98. The method of claim 88, wherein the detecting step includes image analysis of detected ultraviolet light, color change, fluorescence or luminescence.

99. The method of claim 88 wherein one or more steps are automated.

100. The method of claim 88, wherein the steps of the method are performed independently for each test enzyme and corresponding transformed cells.

101. The method of claim 100, wherein one or more steps of the method are preformed contemporaneously for each test enzyme and corresponding transformed cells.

102. The method of claim 88, wherein the steps of the method are repeated until at least one oxygenase enzyme is identified.

103. The method of claim 102, wherein each test enzyme is a variant of at least one identified oxygenase enzyme.

104. The method of claim 88, wherein each transformed cell expresses the coupling enzyme.

105. The method of claim 88, wherein the introducing step comprises providing the transformed cells with a supply of organic substrate and oxygen donor.

106. The method of claim 105, wherein the introducing step comprises providing the transformed cells with a supply of organic substrate and oxygen donor.

107. An oxygenase enzyme variant obtained by the method of claim 1.

108. An oxygenase enzyme variant obtained by the method of claim 20.

109. An oxygenase enzyme variant obtained by the method of claim 47.

110. An oxygenase enzyme variant obtained by the method of claim 58.

111. An oxygenase enzyme variant obtained by the method of claim 65.

112. An oxygenase enzyme variant obtained by the method of claim 88.

113. An oxygenase enzyme variant obtained by the method of claim 103.

114. An oxygenase enzyme variant obtained by the method of claim 104.

115. A P450 oxygenase enzyme variant having at least one mutation at a position corresponding to position 331 of the amino acid sequence of a wild-type P450 enzyme.

116. A P450 oxygenase enzyme variant of claim 115, in which glutamic acid is changed to lysine.

117. A P450 oxygenase enzyme variant having at lease one mutation at a position corresponding to position 280 of the amino acid sequence of a wild-type P450 enzyme.

118. The P450 oxygenase enzyme variant of claim 117, in which arginine is changed to lysine.

119. A P450 oxygenase enzyme variant having at least one mutation at a position corresponding to position 242 of the amino acid sequence of a wild-type P450 enzyme.

120. The P450 oxygenase enzyme variant of claim 119, in which cysteine is changed to phenylalanine.

121. A P450 oxygenase enzyme variant having at least one mutation at a position corresponding to any of positions 242, 280 and 331 of the amino acid sequence of a wild-type P450 enzyme.

122. A P40 oxygenase enzyme variant having at least one mutation in which:

a) glutamic acid is changed to lysine at a position corresponding to position 331 of the amino acid sequence of a wild-type P450 enzyme;

b) arginine is changed to leucine at a position corresponding to position 331 of the amino acid sequence of a wild-type P450 enzyme; and

c) cysteine is changed to phenylalanine at a position corresponding to position 331 of the amino acid sequence of a wild-type P450 enzyme.

123. The sequence-conservative variant of an enzyme of claim 122.

124. The function-conservative variant of an enzyme of claim 122.

125. An oxygenase enzyme variant encoded by a first polynucleotide that hybridizes to a second polypeptide encoded by an enzyme of claim 122 under high stringency conditions.

126. A method for evolving an oxidation enzyme comprising the steps of:

a) supplying an organic substrate and an oxygen donor;

b) providing at least one host cell that expressed a DNA sequence that encodes a provided oxidation enzyme capable of promoting the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

c) generating a test enzyme library comprising a plurality of oxidation enzyme mutants, each of which is a variant of at least one provided DNA sequence;

d) expressing a plurality of variants in host cells to produce a plurality of test enzymes from the library;

e) introducing each test enzyme to the organic substrate and the oxygen donor;

f) furnishing a coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product;

g) testing for the detectable composition, wherein the presence of the detectable composition identifies the test enzyme as an oxidation enzyme;

h) selecting at least one identified oxidation enzyme by comparison with at least one provided oxidation enzyme according to at least one property.

127. The method of claim 126, wherein the method is repeated, using at least one identified oxidation enzyme as a provided oxidation enzyme.

128. The method of claim 126, wherein the generating step includes the use of error-prone PCR to provide mutants.

129. The method according to claim 126, wherein the oxygen donor is hydrogen peroxide, and the selecting step includes a comparison of enzyme activity.

130. An P450 enzyme evolved according to claim 129, and having an improved enzyme activity or stability in comparison with a provided P450 enzyme.

131. A P450 enzyme for use with hydrogen peroxide and obtained by the method of claim 126.

132. A method for evaluating the reaction conditions for an oxidation catalyst comprising the steps of:

a) providing an oxidation catalyst;

b) supplying an organic substrate and an oxygen donor;

c) introducing the catalyst to the organic substrate and the oxygen donor under each of a plurality of reaction conditions, to form a plurality of test combinations;

d) furnishing each test combination with a coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product;

e) examining the test combinations for production of the detectable composition, wherein the reaction conditions are evaluated according to differences in at least one of the relative rates and amounts of production of the detectable composition.

133. An evolved P450 enzyme variant having a catalytic activity at least twice as active as a corresponding P450 wild-type enzyme in facilitating the oxidation of a substrate in the presence of hydrogen peroxide.

134. An evolved P450 enzyme variant having a catalytic activity at least ten times as active as a corresponding P450 wild-type enzyme in facilitating the oxidation of a substrate in the presence of hydrogen peroxide.

135. An evolved P450 enzyme variant having a catalytic activity at least twice as stable as a corresponding P450 wild-type enzyme.

136. An evolved P450 enzyme variant having a catalytic activity at least ten times as stable as a corresponding P450 wild-type enzyme.

137. A method for detecting an oxidation catalyst comprising the steps of:

a) providing an organic substrate and an oxygen donor;

b) supplying a test catalyst selected for evaluation of its ability to influence the formation of an oxygen-containing product from the organic substrate and the oxygen donor;

c) introducing the test catalyst to the organic substrate and the oxygen donor;

d) furnishing a coupling enzyme selected to promote the formation of a detectable composition from the oxygen-containing product; and

e) testing for the detectable composition, wherein the presence of the detectable composition indicates that the test catalyst is an oxidation catalyst.

138. The method of claim 137, wherein the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin.

139. The method of claim 1, wherein the oxygen donor is a peroxide.

140. The method of claim 137, wherein the coupling enzyme is used to bring together molecules of the oxygen-containing product to form a polymeric composition that is detectable using at least one of ultraviolet light, a color change, fluorescence and luminescence.

141. The method of claim 137, wherein the coupling enzyme is at least one of a peroxidase enzyme and a laccase enzyme.

142. The method of claim 137, wherein the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and peroxidase Novozyme® 502.

143. The method of claim 137, wherein the organic substrate is an aromatic compound, the oxygen donor is a peroxide and the coupling enzyme is a peroxidase enzyme.

144. The method of claim 137, further comprising the step of providing a chemiluminescent agent.

145. The method of claim 137, wherein a plurality of indicated oxidation catalysts are compared to each other by evaluating the corresponding testing for detectable composition.

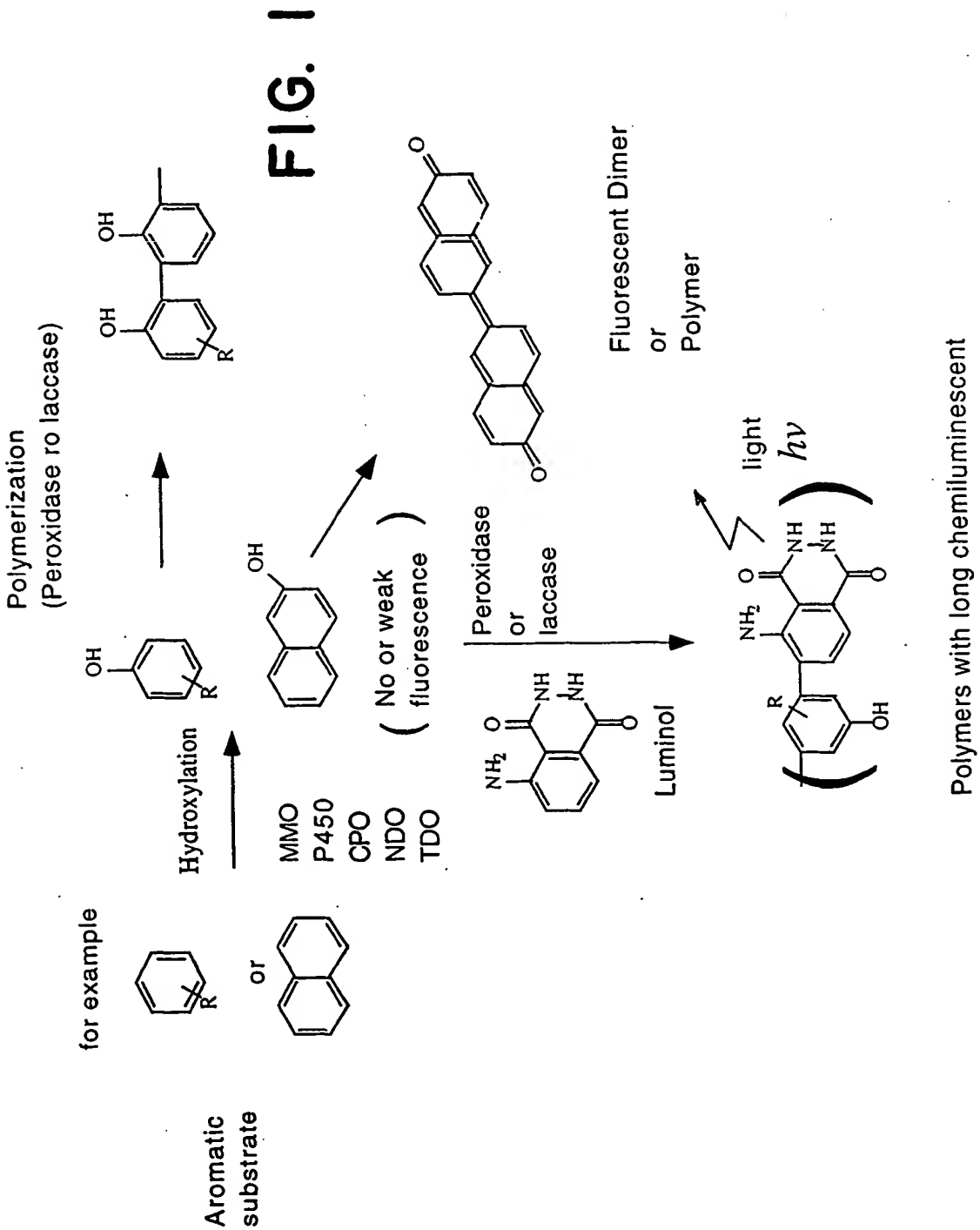

Aromatic substrate
for example
or
Hydroxylation
MMO
P450
CPO
NDO
TDO
No or weak fluorescence
Polymerization
(Peroxidase ro laccase)
Peroxidase
or
laccase
Luminol
Fluorescent Dimer
or
Polymer
light
hν
Polymers with long chemiluminescent

FIG. 1

FIG. 2

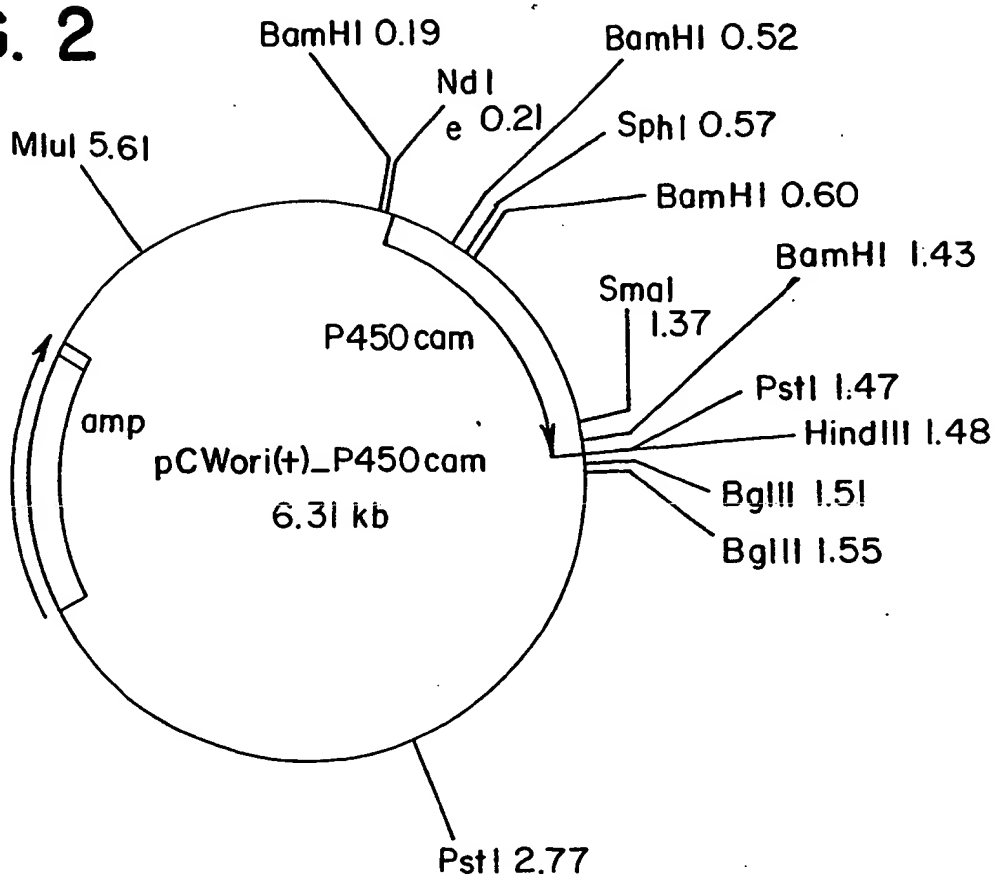

BamHI 0.19
BamHI 0.52
Nde I
Sph I 0.57
MluI 5.61
BamHI 0.60
BamHI 1.43
SmaI
1.37
P450cam
PstI 1.47
amp
HindIII 1.48
pCWori(+)_P450cam
6.31 kb
BglII 1.51
BglII 1.55
PstI 2.77

FIG. 16

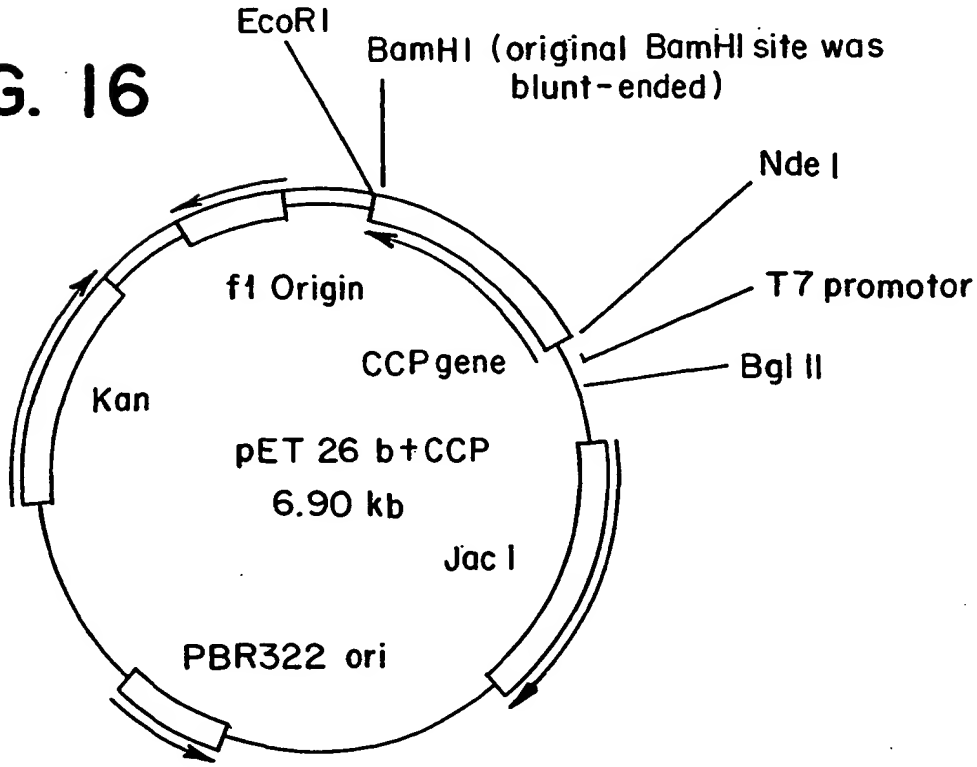

EcoRI
BamHI (original BamHI site was blunt-ended)
Nde I
f1 Origin
T7 promotor
CCP gene
Bgl II
Kan
pET 26 b+CCP
6.90 kb
Jac I
PBR322 ori

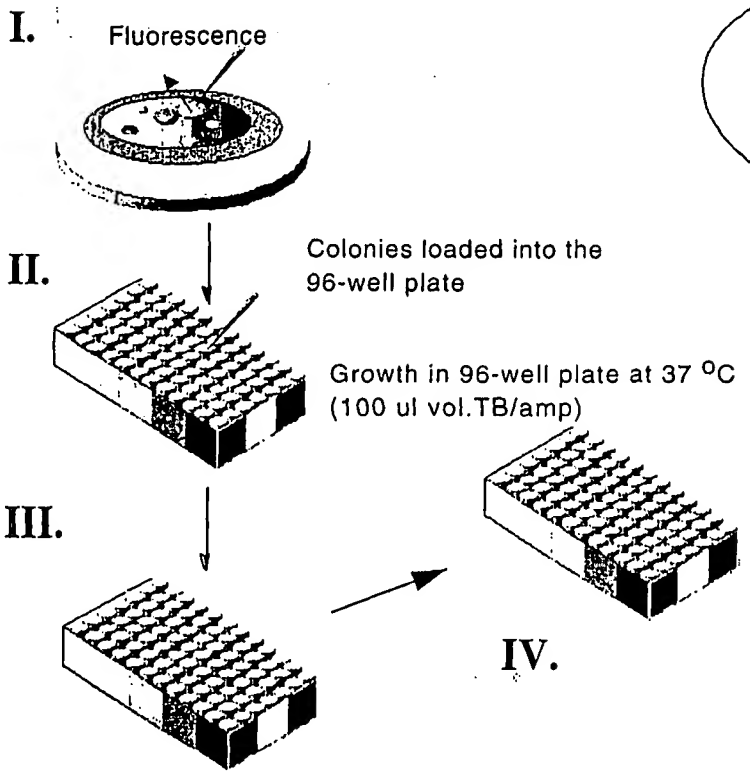
FIG. 3A

```
   1 CTGCAGGATC GTTATCCGCT GGCCGATCTG ATCACCCAGC GTTTTTCCAT CGACGAGGCC
  61 AGCAAGGCAC TTGAACTGGT CAAGGCAGGA GCACTGATCA AACCCGTGAT CGACTCCACT
 121 CTTTAGCCAA CCCGCGTTCC AGGAGAACAA CAACAATGAC GACTGAAACC ATACAAAGCA
 181 ACGCCAATCT TGCCCCTCTG CCACCCCATG TGCCAGAGCA CCTGGTATTC GACTTCGACA
 241 TGTACAATCC GTCGAATCTG TCTGCCGGCG TGCAGGAGGC CTGGGCAGTT CTGCAAGAAT
 301 CAAACGTACC GGATCTGGTG TGGACTCGCT GCAACGGCGG ACACTGGATC GCCACTCGCG
 361 GCCAACTGAT CCGTGAGGCC TATGAAGATT ACCGCCACTT TTCCAGCGAG TGCCCGTTCA
 421 TCCCTCGTGA AGCCGGCGAA GCCTACGACT TCATTCCCAC CTCGATGGAT CCGCCCGAGC
 481 AGCGCCAGTT TCGTGCGCTG GCCAACCAAG TGGTTGGCAT GCCGGTGGTG GATAAGCTGG
 541 AGAACCGGAT CCAGGAGCTG GCCTGCTCGC TGATCGAGAG CCTGCGCCCG CAAGGACAGT
 601 GCAACTTCAC CGAGGACTAC GCCGAACCCT TCCCGATACG CATCTTCATG CTGCTCGCAG
 661 GTCTACCGGA AGAAGATATC CCGCACTTGA AATACCTAAC GGATCAGATG ACCCGTCCGG
 721 ATGGCAGCAT GACCTTCGCA GAGGCCAAGG AGGCGCTCTA CGACTATCTG ATACCGATCA
 781 TCGAGCAACG CAGGCAGAAG CCGGGAACCG ACGCTATCAG CATCGTTGCC AACGGCCAGG
 841 TCAATGGGCG ACCGATCACC AGTGACGAAG CCAAGAGGAT GTGTGGCCTG TTACTGGTCG
 901 GCGGCCTGGA TACGGTGGTC AATTTCCTCA GCTTCAGCAT GGAGTTCCTG GCCAAAAGCC
 961 CGGAGCATCG CCAGGAGCTG ATCGAGCGTC CCGAGCGTAT TCCAGCCGCT TGCGAGGAAC
1021 TACTCCGGCG CTTCTCGCTG GTTGCCGATG GCGCATCCT CACCTCCGAT TACGAGTTTC
1081 ATGGCGTGCA ACTGAAGAAA GGTGACCAGA TCCTGCTACC GCAGATGCTG TCTGGCCTGG
1141 ATGAGCGCGA AAACGCCTGC CCGATGCACG TCGACTTCAG TCGCCAAAAG GTTTCACACA
1201 CCACCTTTGG CCACGGCAGC CATCTGTGCC TTGGCCAGCA CCTGGCCCGC CGGGAAATCA
1261 TCGTCACCCT CAAGGAATGG CTGACCAGGA TTCCTGACTT CTCCATTGCC CCGGGTGCCC
1321 AGATTCAGCA CAAGAGCGGC ATCGTCAGCG GCGTGCAGGC ACTCCCTCTG GTCTGGGATC
1381 CGGCGACTAC CAAAGCGGTA TA
```

FIG. 3B

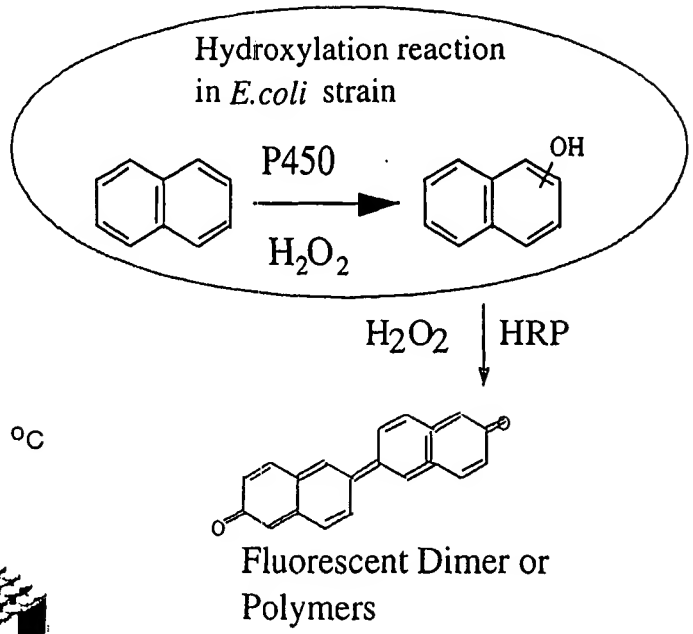
THR THR GLU THR ILE GLN SER ASN ALA ASN LEU ALA PRO
LEU PRO PRO HIS VAL PRO GLU HIS LEU VAL PHE ASP PHE
ASP MET TYR ASN PRO SER ASN LEU SER ALA GLY VAL GLN
GLU ALA TRP ALA VAL LEU GLN GLU SER ASN VAL PRO ASP
LEU VAL TRP THR ARG CYS ASN GLY GLY HIS TRP ILE ALA
THR ARG GLY GLN LEU ILE ARG GLU ALA TYR GLU ASP TYR
ARG HIS PHE SER SER GLU CYS PRO PHE ILE PRO ARG GLU
ALA GLY GLU ALA TYR ASP PHE ILE PRO THR SER MET ASP
PRO PRO GLU GLN ARG GLN PHE ARG ALA LEU ALA ASN GLN
VAL VAL GLY MET PRO VAL VAL ASP LYS LEU GLU ASN ARG
ILE GLN GLU LEU ALA CYS SER LEU ILE GLU SER LEU ARG
PRO GLN GLY GLN CYS ASN PHE THR GLU ASP TYR ALA GLU
PRO PHE PRO ILE ARG ILE PHE MET LEU LEU ALA GLY LEU
PRO GLU GLU ASP ILE PRO HIS LEU LYS TYR LEU THR ASP
GLN MET THR ARG PRO ASP GLY SER MET THR PHE ALA GLU
ALA LYS GLU ALA LEU TYR ASP TYR LEU ILE PRO ILE ILE
GLU GLN ARG ARG GLN LYS PRO GLY THR ASP ALA ILE SER
ILE VAL ALA ASN GLY GLN VAL ASN GLY ARG PRO ILE THR
SER ASP GLU ALA LYS ARG MET CYS GLY LEU LEU LEU VAL
GLY GLY LEU ASP THR VAL VAL ASN PHE LEU SER PHE SER
MET GLU PHE LEU ALA LYS SER PRO GLU HIS ARG GLN GLU
LEU ILE GLU ARG PRO GLU ARG ILE PRO ALA ALA CYS GLU
GLU LEU LEU ARG ARG PHE SER LEU VAL ALA ASP GLY ARG
ILE LEU THR SER ASP TYR GLU PHE HIS GLY VAL GLN LEU
LYS LYS GLY ASP GLN ILE LEU LEU PRO GLN MET LEU SER
GLY LEU ASP GLU ARG GLU ASN ALA CYS PRO MET HIS VAL
ASP PHE SER ARG GLN LYS VAL SER HIS THR THR PHE GLY
HIS GLY SER HIS LEU CYS LEU GLY GLN HIS LEU ALA ARG
ARG GLU ILE ILE VAL THR LEU LYS GLU TRP LEU THR ARG
ILE PRO ASP PHE SER ILE ALA PRO GLY ALA GLN ILE GLN
HIS LYS SER GLY ILE VAL SER GLY VAL GLN ALA LEU PRO
LEU VAL TRP ASP PRO ALA THR THR LYS ALA VAL

FIG. 4A



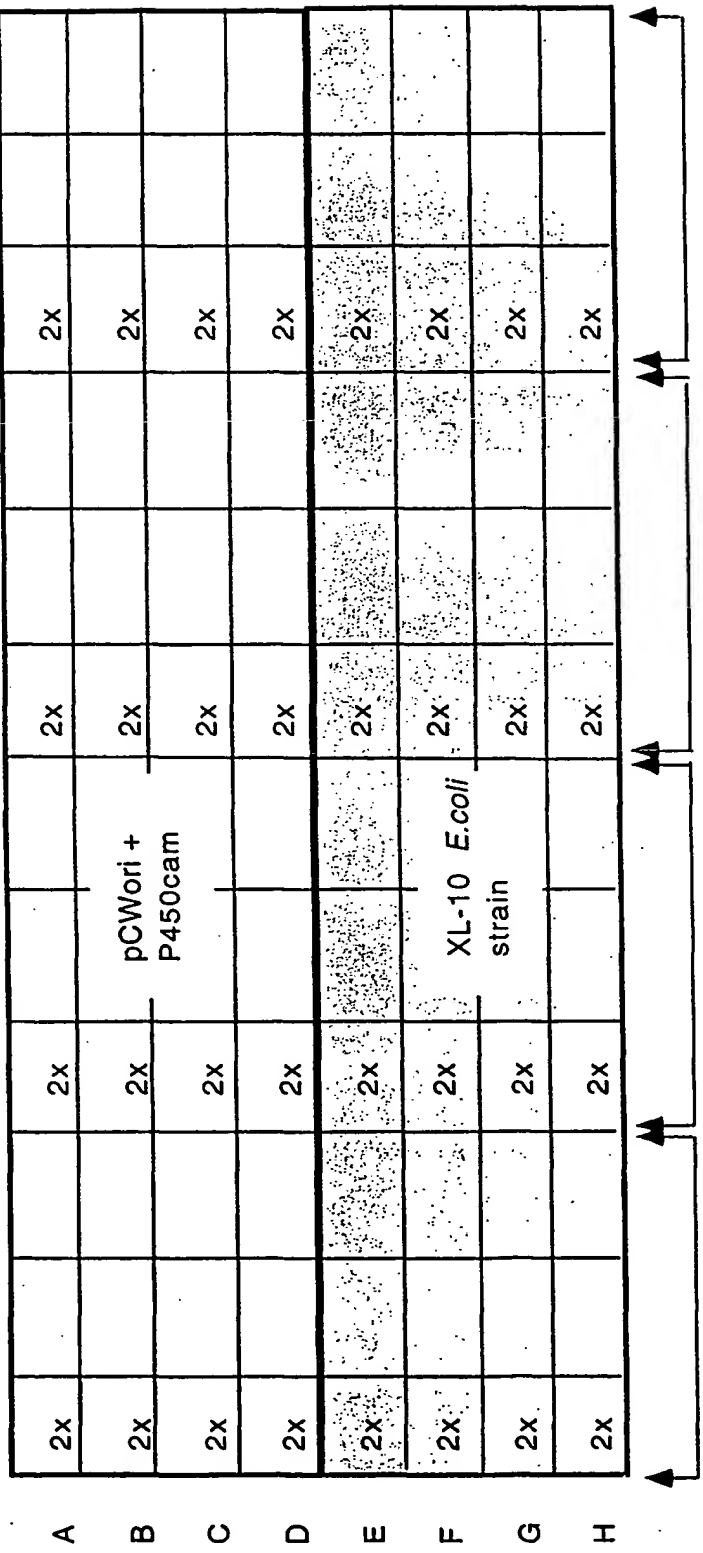
P450cam protein expression by adding 0.5-1 mM IPTG and 1 mM thaimine, 0.5 -1.3 mM delta-ALA, 0.5 ml trace element stock/10ml medium (total : 120 ul. vol)
Induction time : 24 hours
Induction temperature : 30 °C

FIG. 4B



Reaction mixture (total 200 ul vol.):
- 50 ul culture broth
- 100 ul sodium dibasic phosphate buffer (50 mM, pH 9.0)
- 10 ul pure ethanol
- 20 ul naphthalene stock (1 g/13 ml ion pure ethanol)
- 10 ul hydrogen peroxide stock solution (100 mM)
- 10 ul horseradish peroxidase stock solution (1400 unitsa/10 ml)

FIG. 5A



* 2x : 200 ul cultivation volume, others : 100 ul cultivation volume

FIG. 5B

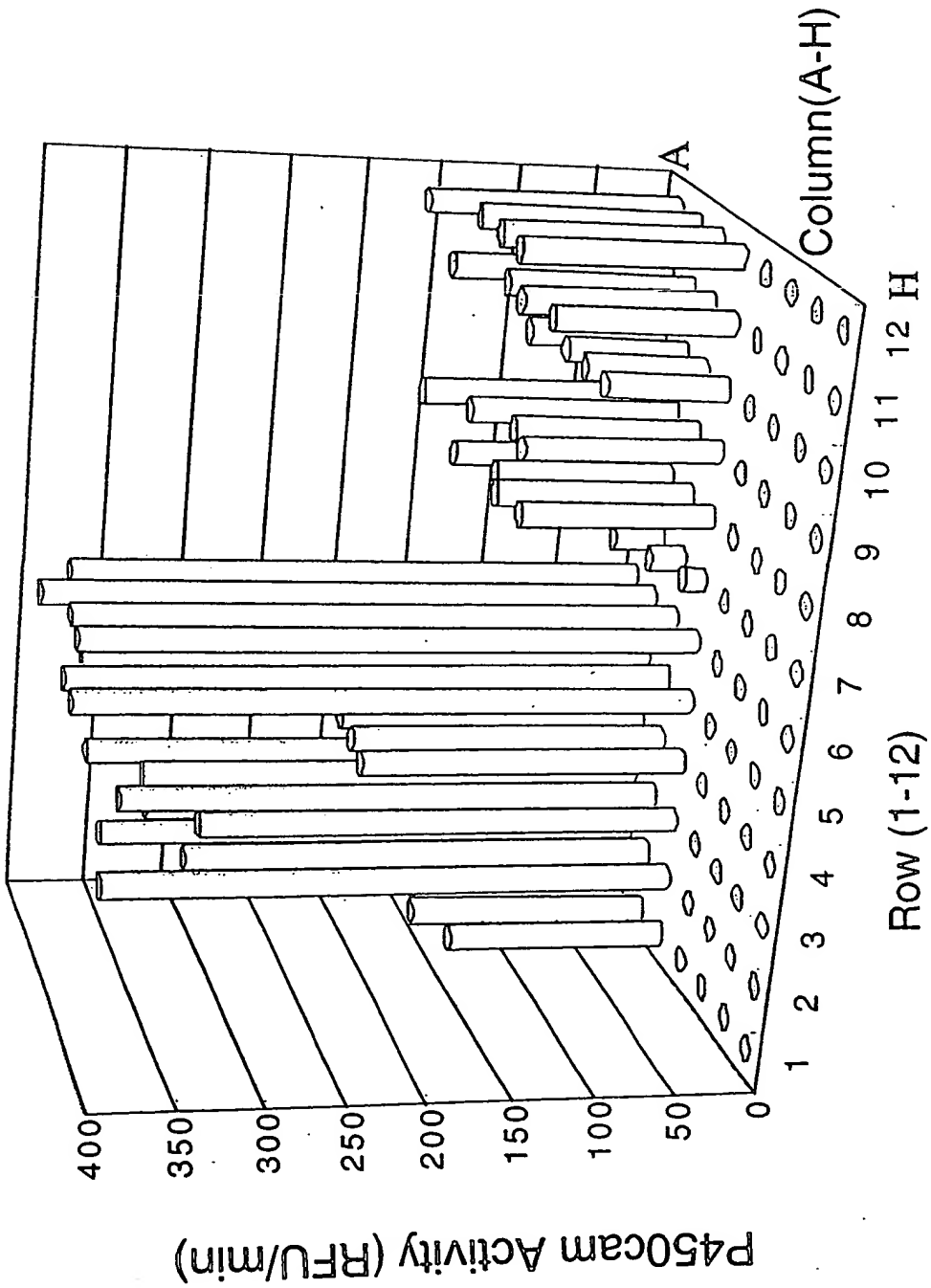
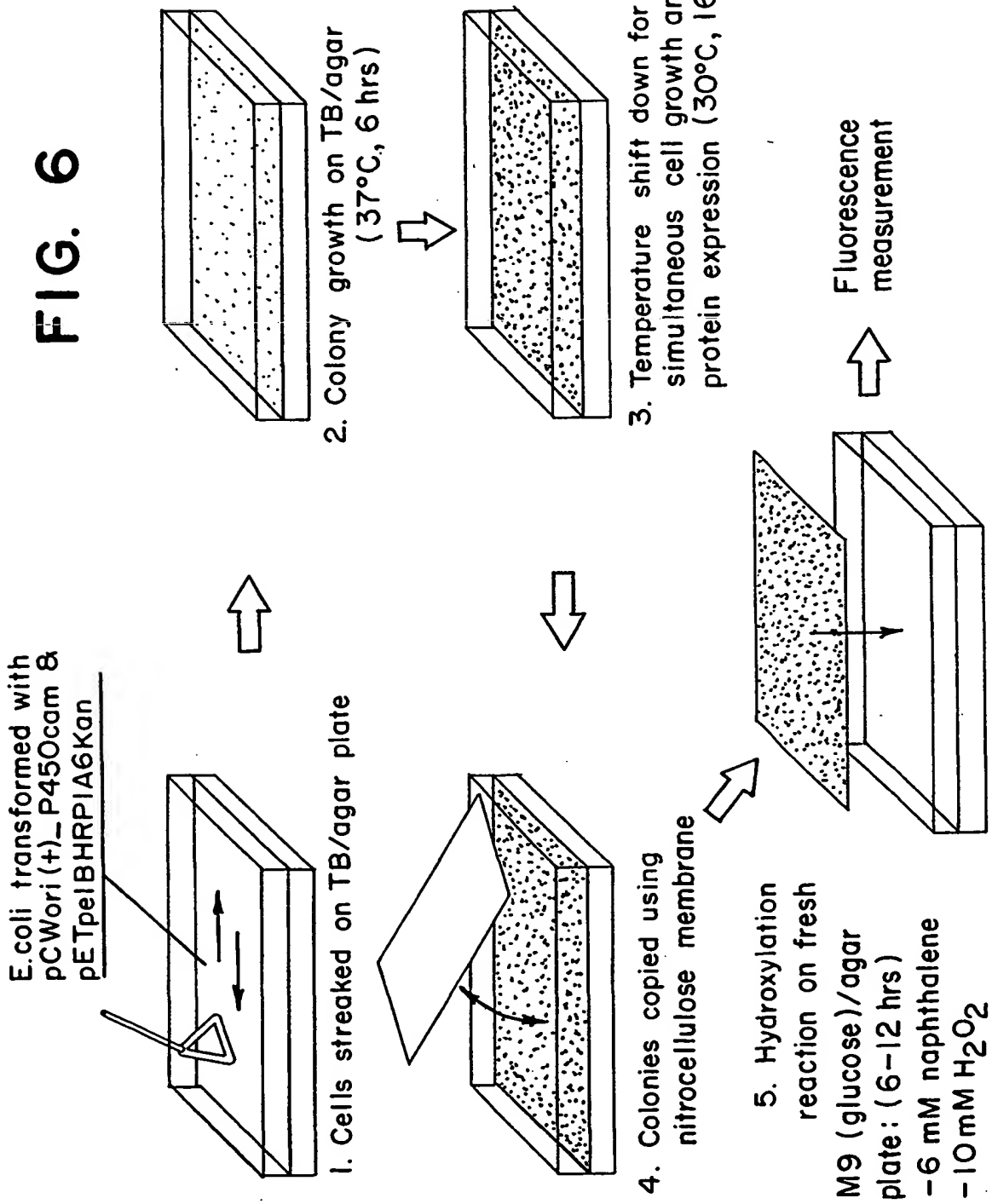

P450cam Activity (RFU/min)
400
350
300
250
200
150
100
50
0
Row (1-12)
1 2 3 4 5 6 7 8 9 10 11 12
Column(A-H)
A
H

FIG. 6


E.coli transformed with
pCWori(+)_P450cam &
pETpelBHRPIA6Kan
1. Cells streaked on TB/agar plate
2. Colony growth on TB/agar
(37°C, 6 hrs)
3. Temperature shift down for
simultaneous cell growth and
protein expression (30°C, 16 hrs.)
4. Colonies copied using
nitrocellulose membrane
5. Hydroxylation
reaction on fresh
M9 (glucose)/agar
plate: (6-12 hrs)
-6 mM naphthalene
-10 mM H_2O_2
Fluorescence
measurement

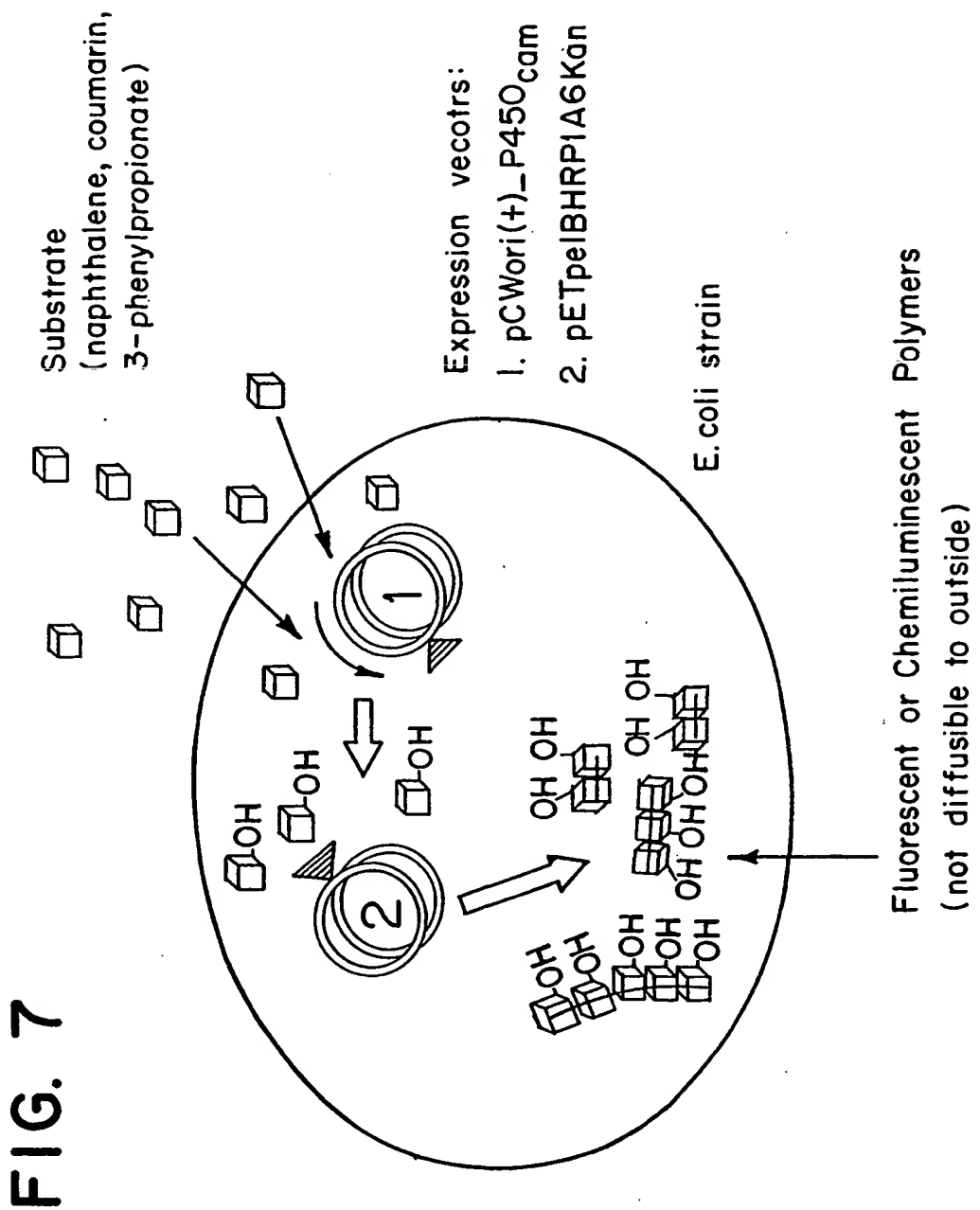

Substrate
(naphthalene, coumarin,
3-phenylpropionate)
Expression vecotrs:
1. pCWori(+)_P450cam
2. pETpelBHRP1A6Kan
E. coli strain
Fluorescent or Chemiluminescent Polymers
(not diffusible to outside)
1
2
OH

FIG. 7

FIG. 8

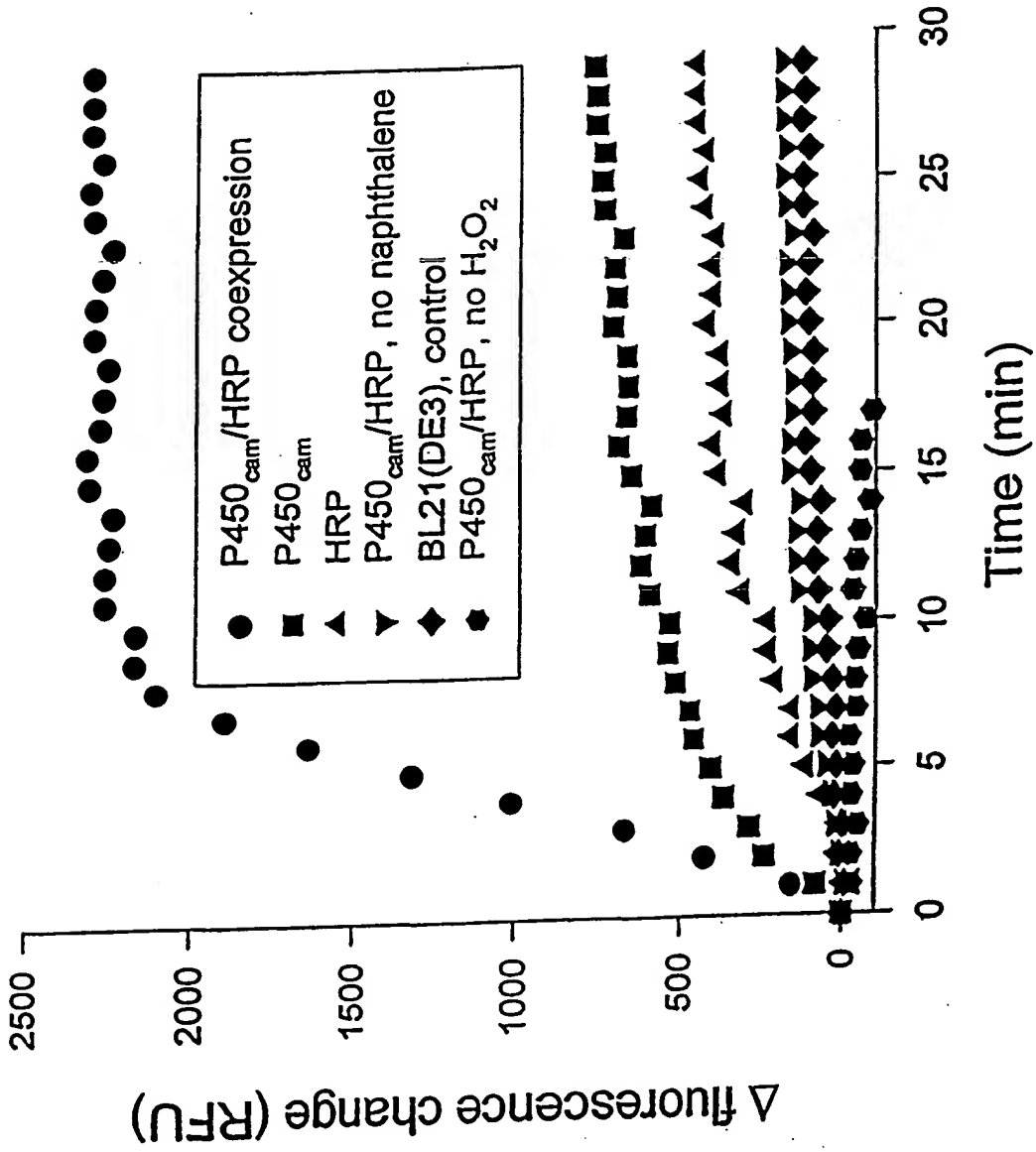

P450cam/HRP coexpression
P450cam
HRP
P450cam/HRP, no naphthalene
BL21(DE3), control
P450cam/HRP, no H2O2
Δ fluorescence change (RFU)
Time (min)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30

FIG. 9

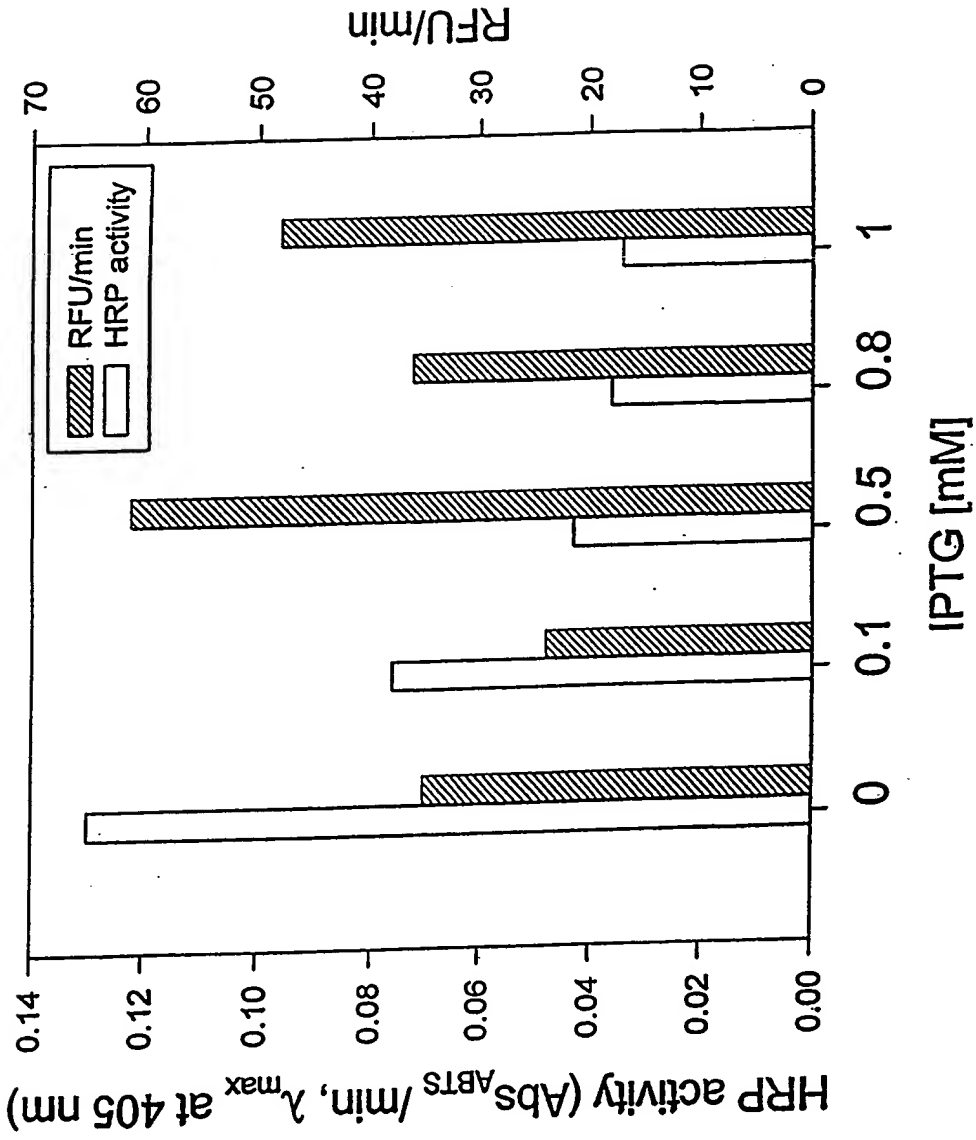


FIG. 10

FIG. 12A

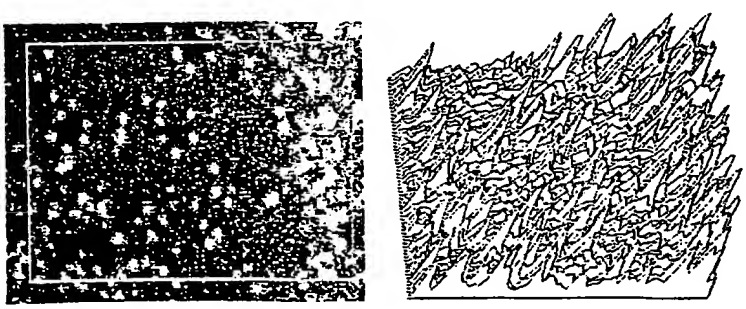
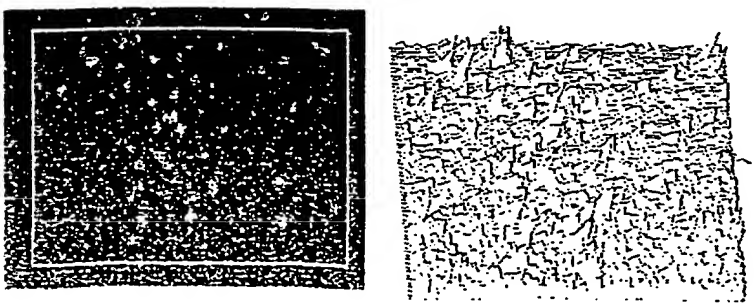


FIG. 12B



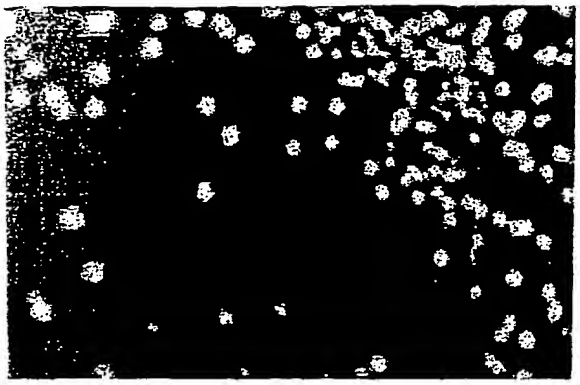


FIG. IIA

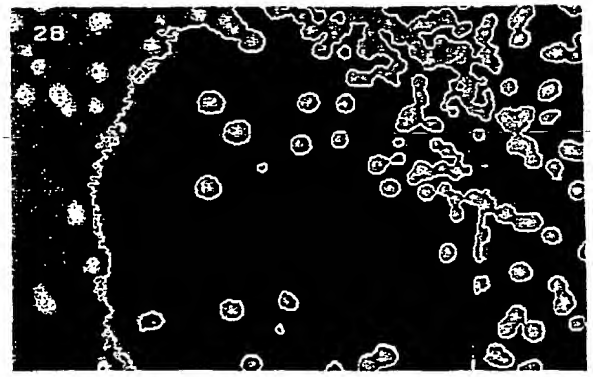


FIG. IIB

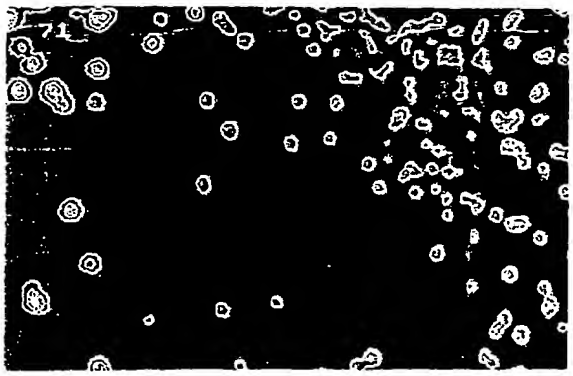


FIG. IIC

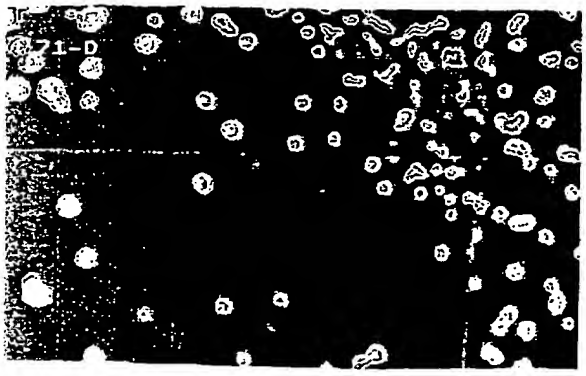


FIG. IID

FIG. 12C

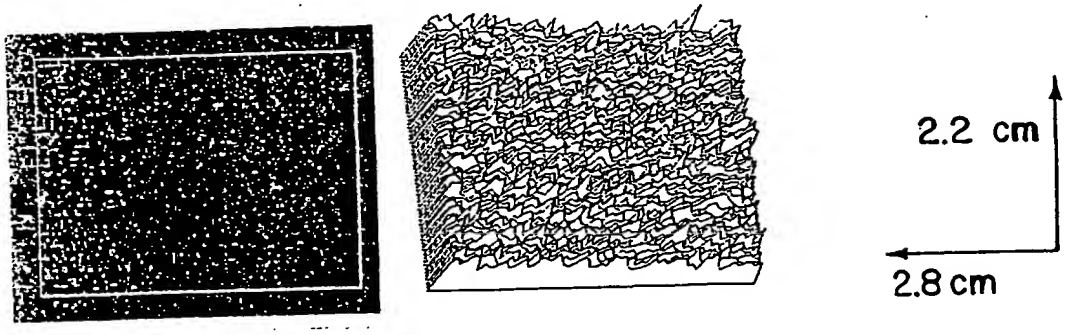


FIG. 12D

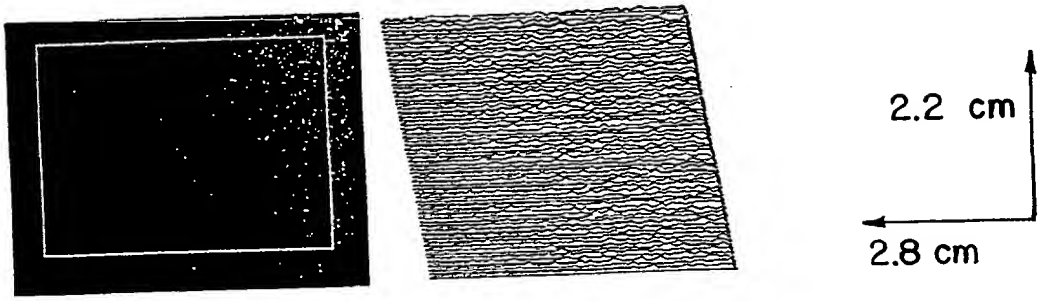


FIG. 12E

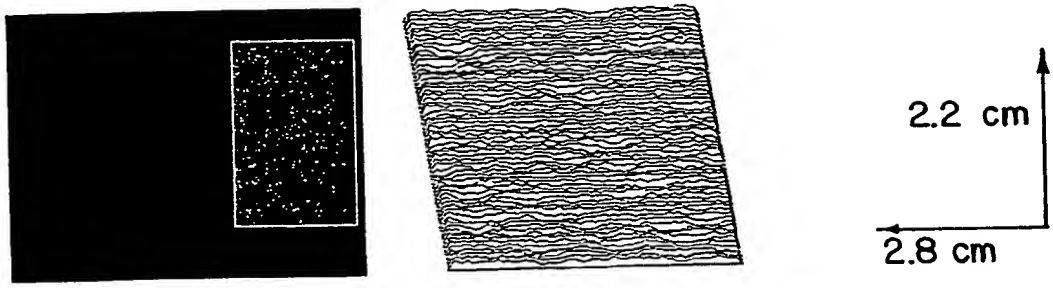


FIG. 12F

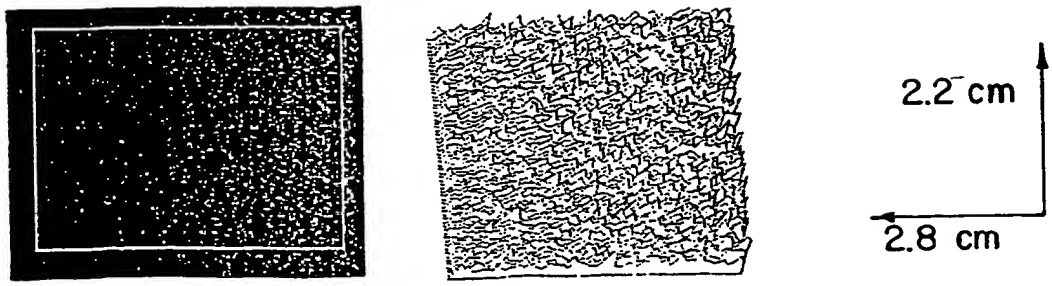


FIG. 13

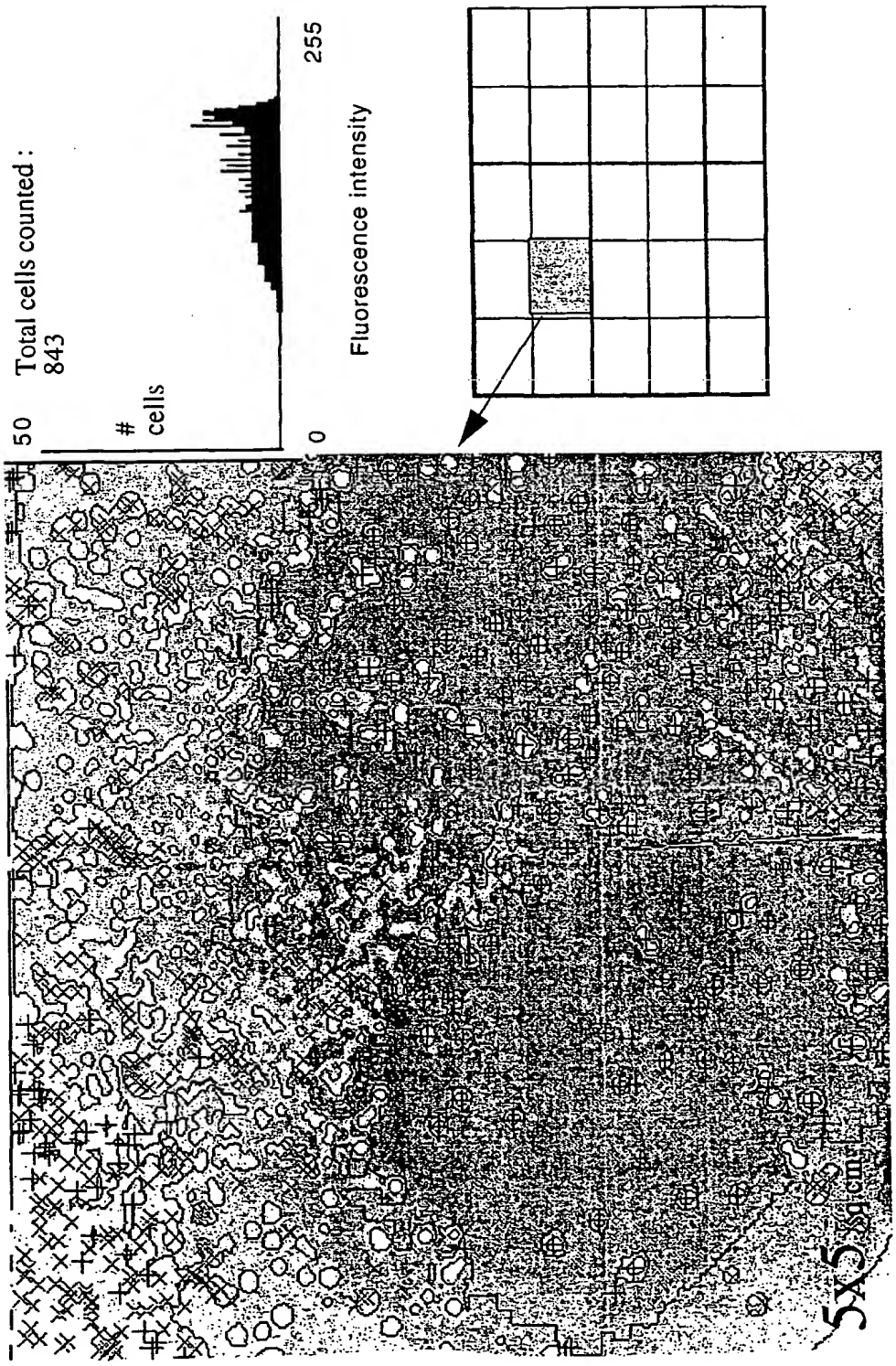

Total cells counted :
843
cells
50
0
255
Fluorescence intensity
5X5 sq cm

FIG. 14A

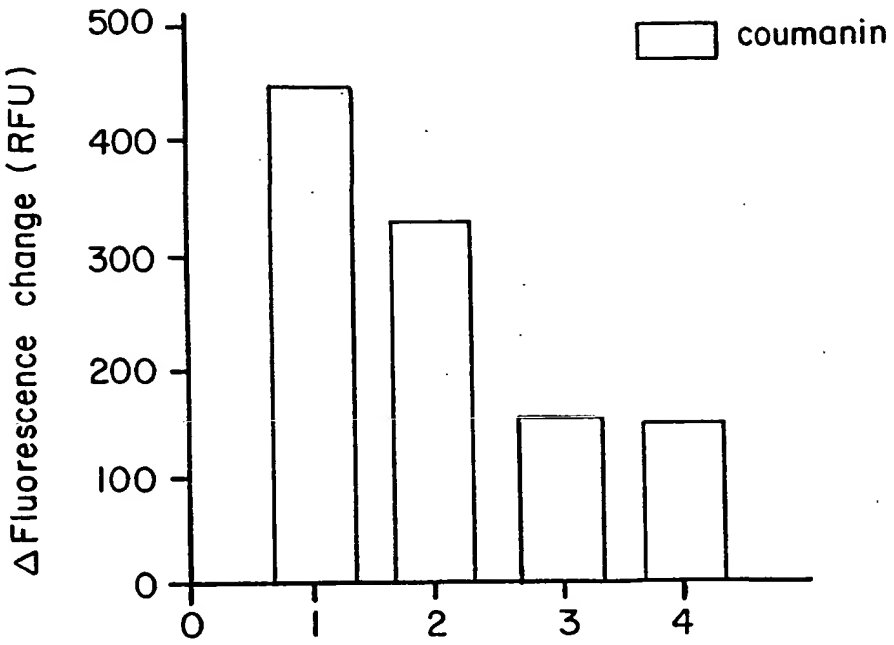


FIG. 14B

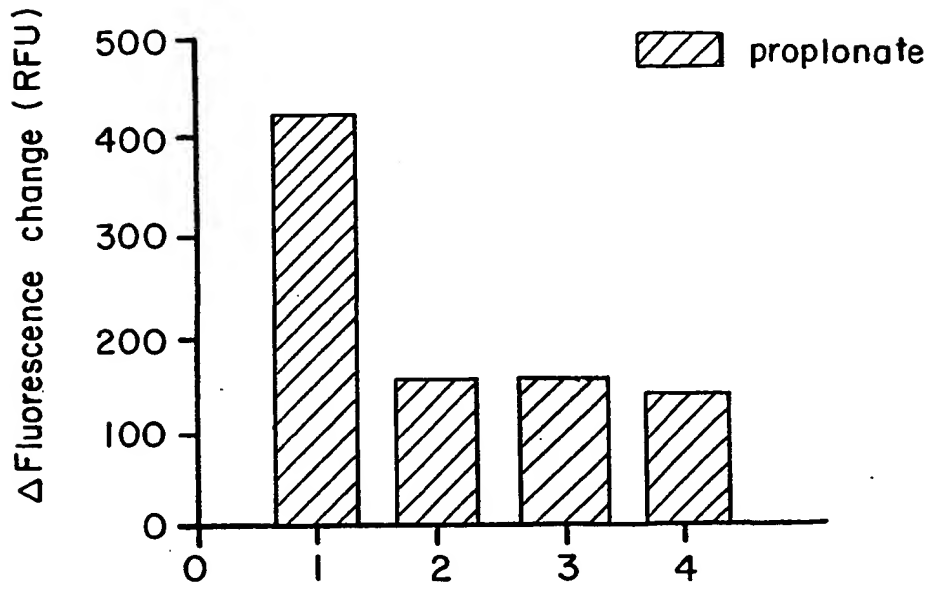
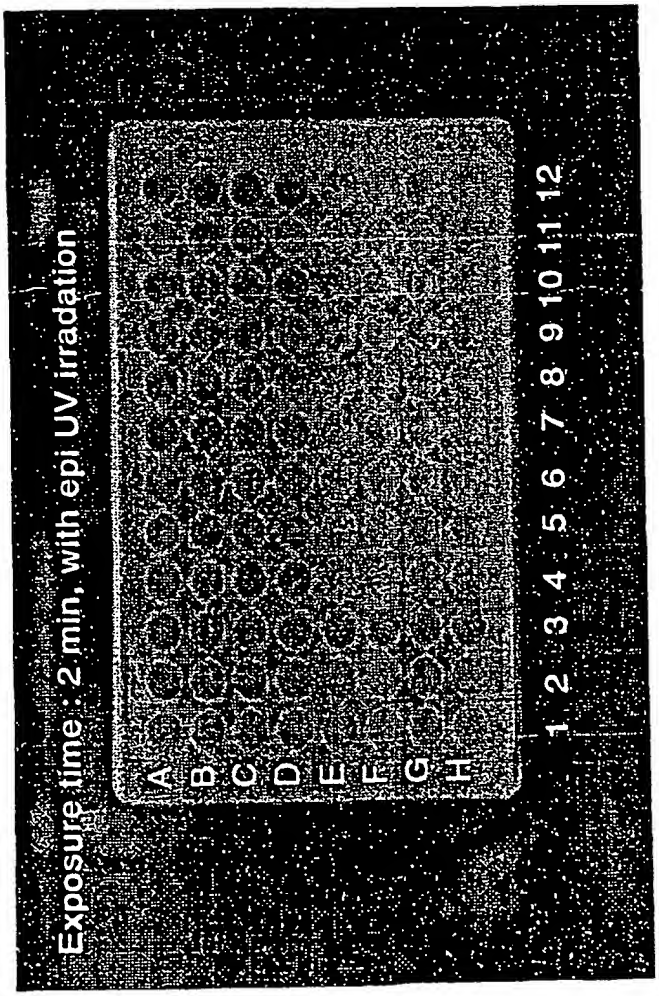


FIG. 15A



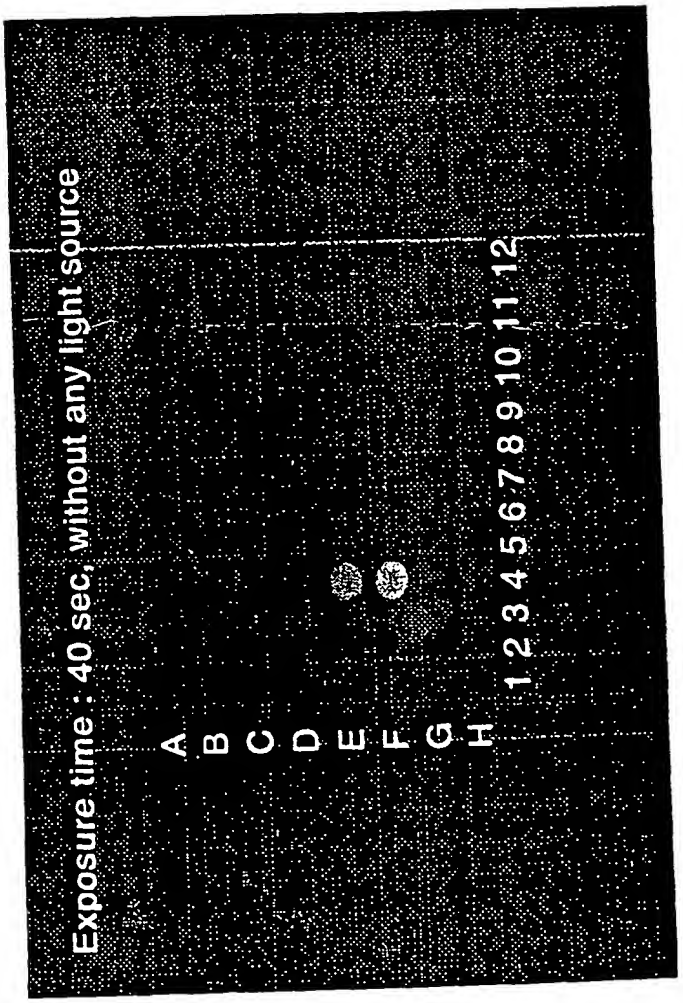
Row:

E: 60uM luminol+0.5 mM PPP
F: 120 uM luminol+0.5 mM PPP
G: 60 uM luminol
H: 120 uM luminol

Column:

4: P450cam/HRP1A6 in BL21 (DE3)
5: P450cam in BL21 (DE3)
6: HRP1A6 in BL21 (DE3)
7: Host strain, BL21 (DE3)

FIG. 15B



Light Emission Values

E4: 51 ILDV
F4: 98 ILDV
G4: 0.2 ILDV
H4: 1 ILDV

Others: <0.1 ILDV

FIG. 17

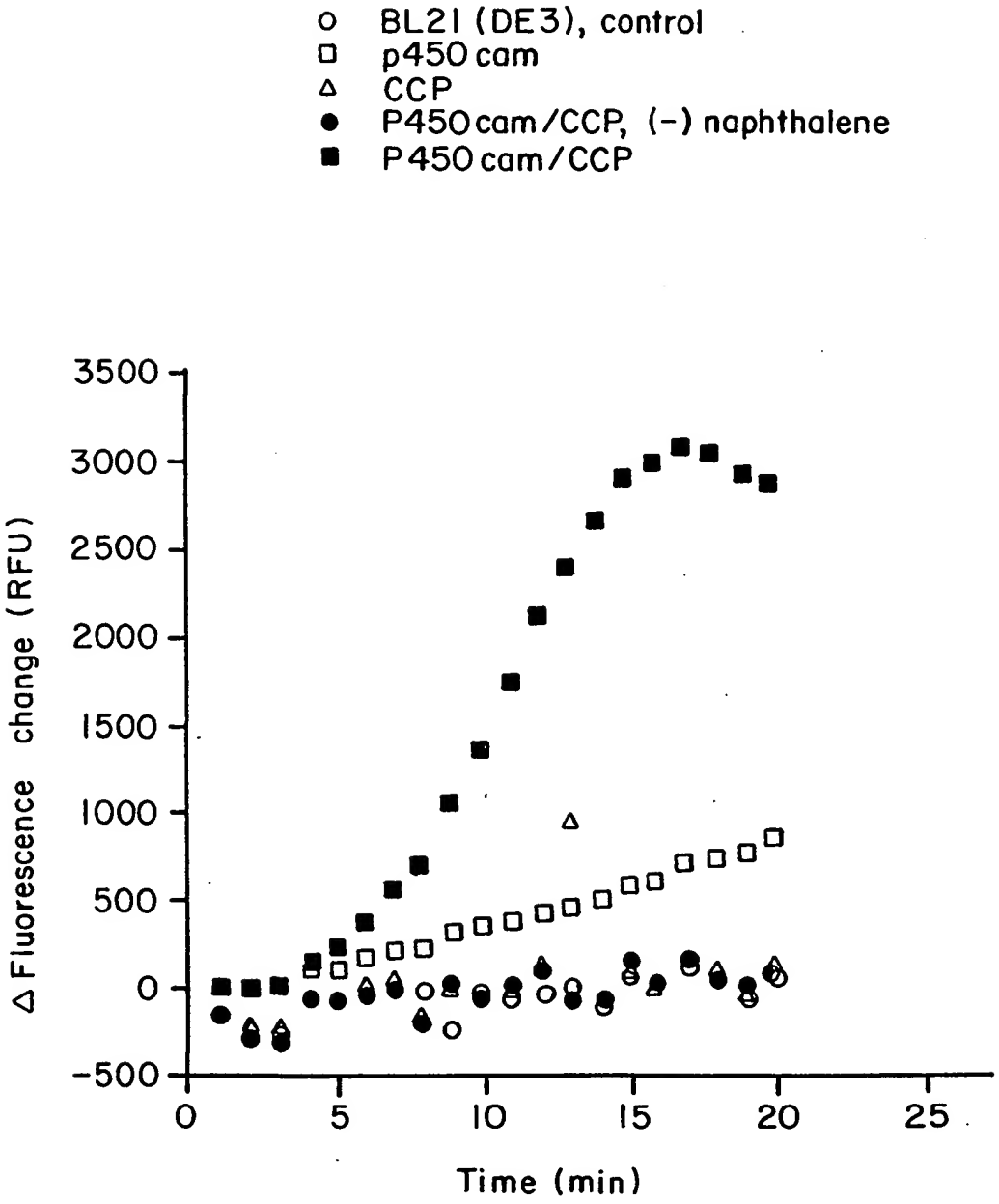

BL21 (DE3), control
p450 cam
CCP
P450 cam/CCP, (-) naphthalene
P450 cam/CCP
Δ Fluorescence change (RFU)
3500
3000
2500
2000
1500
1000
500
0
-500
0
5
10
15
20
25
Time (min)

FIG. 18

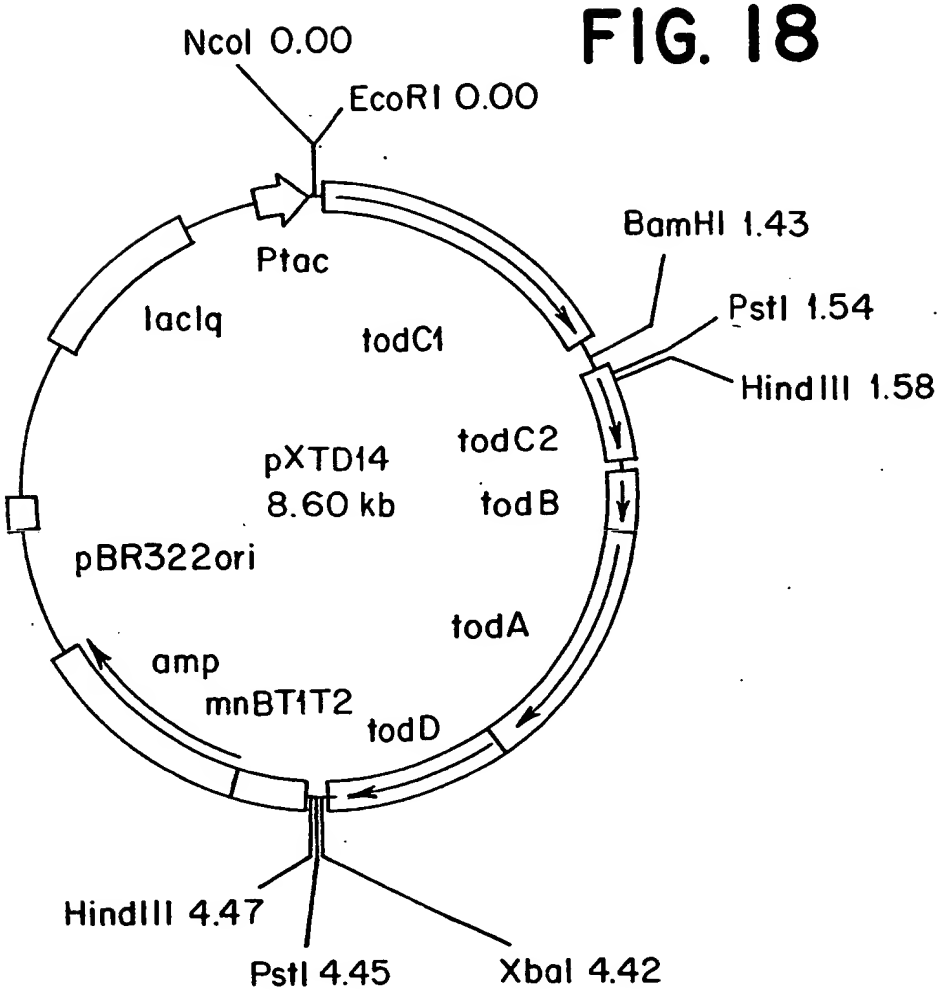

NcoI 0.00
EcoRI 0.00
BamHI 1.43
PstI 1.54
HindIII 1.58
Ptac
lacIq
todC1
todC2
pXTD14
8.60 kb
todB
pBR322ori
todA
amp
mnBT1T2
todD
HindIII 4.47
PstI 4.45
XbaI 4.42

FIG. 21

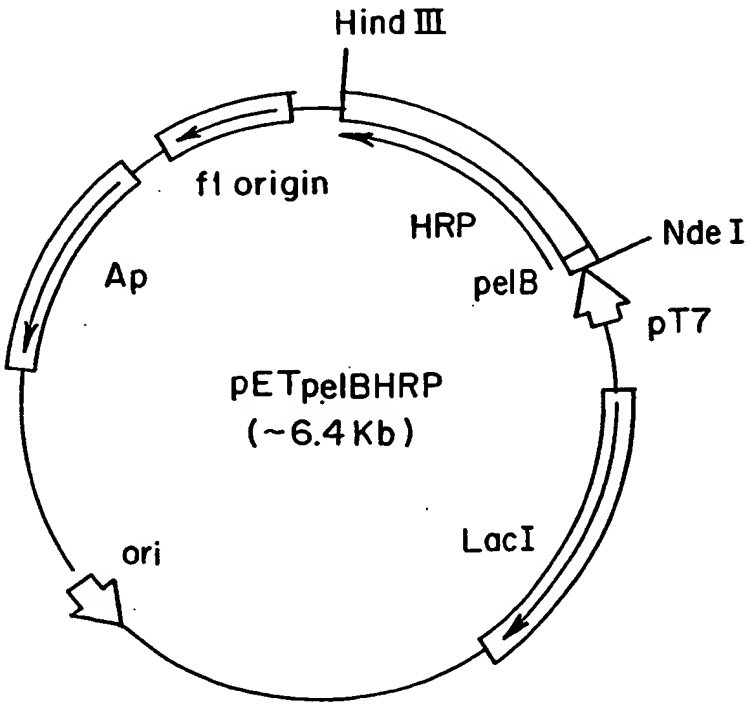

Hind III
f1 origin
HRP
Nde I
Ap
pelB
pT7
pETpelBHRP
(~6.4 Kb)
ori
LacI

FIG. 19A

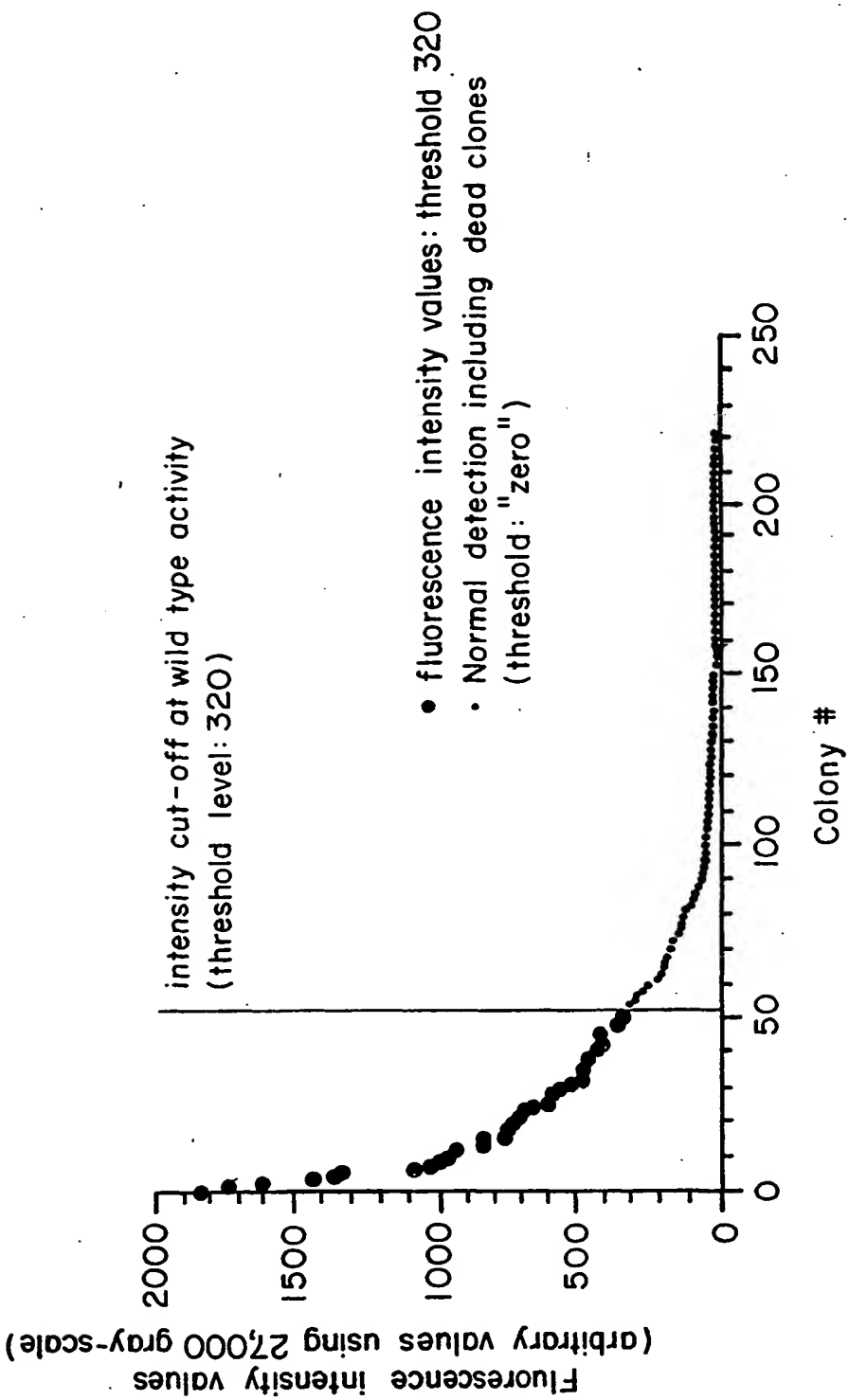

Fluorescence intensity values
(arbitrary values using 27,000 gray-scale)
2000
1500
1000
500
0
0
50
100
150
200
250
Colony #
intensity cut-off at wild type activity
(threshold level: 320)
• fluorescence intensity values: threshold 320
• Normal detection including dead clones
(threshold: "zero")

FIG. 19B

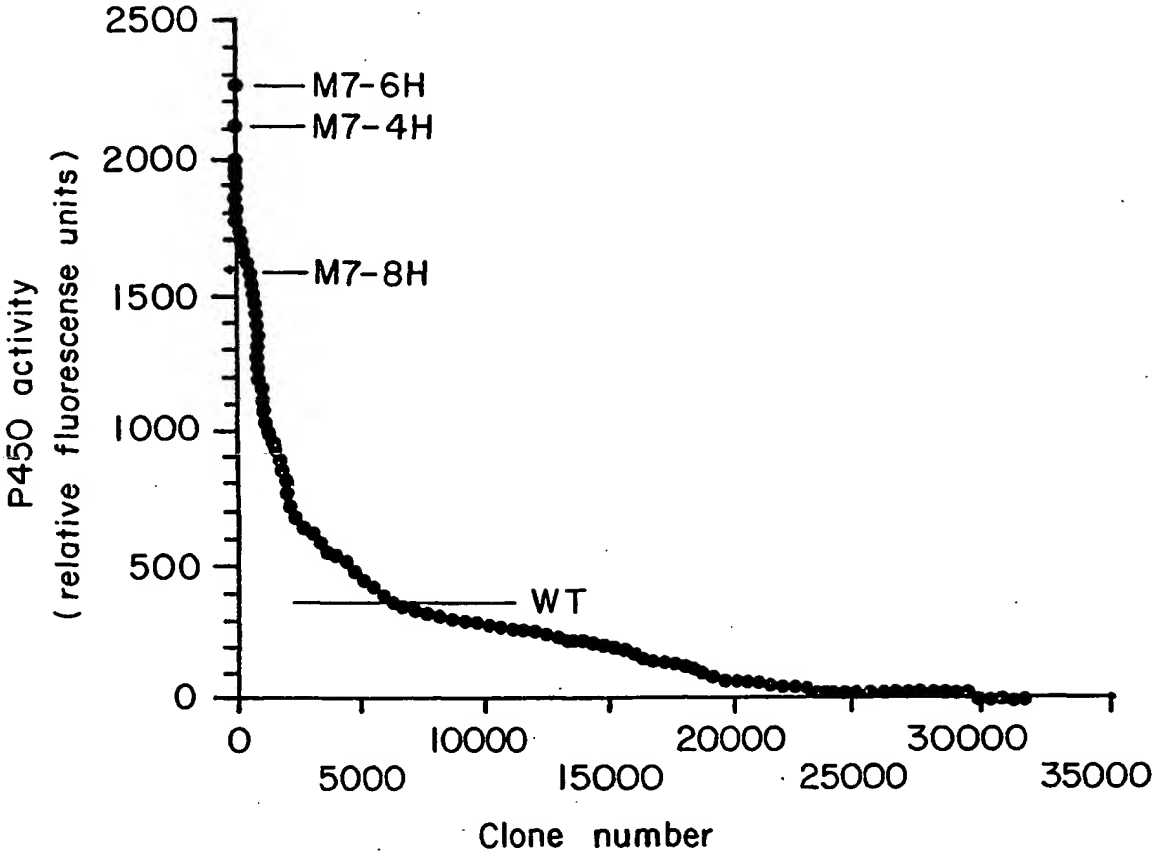


FIG. 19C

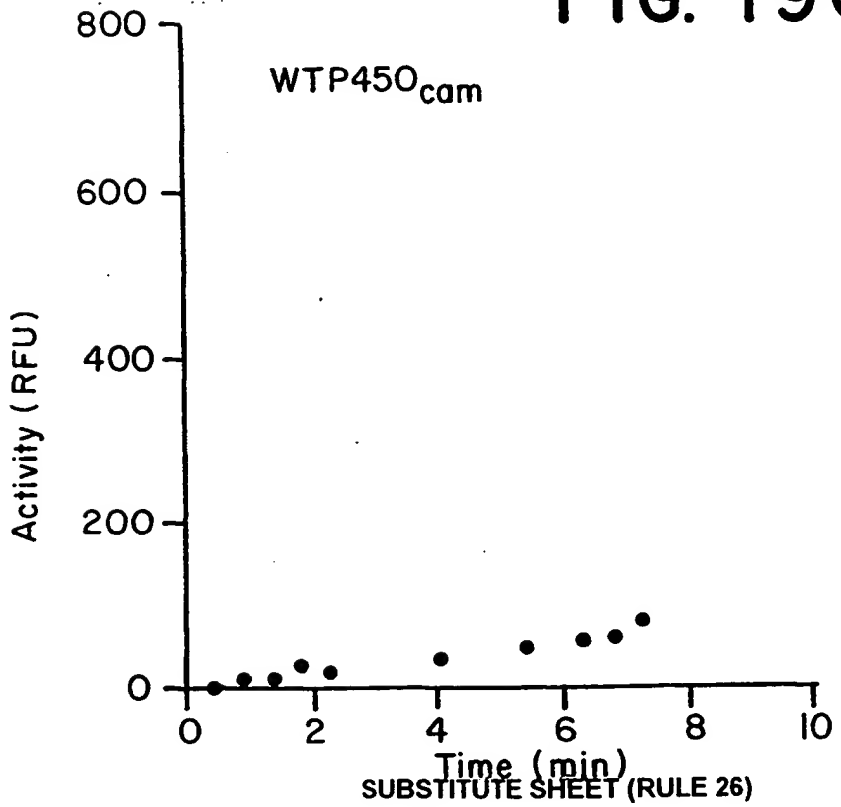
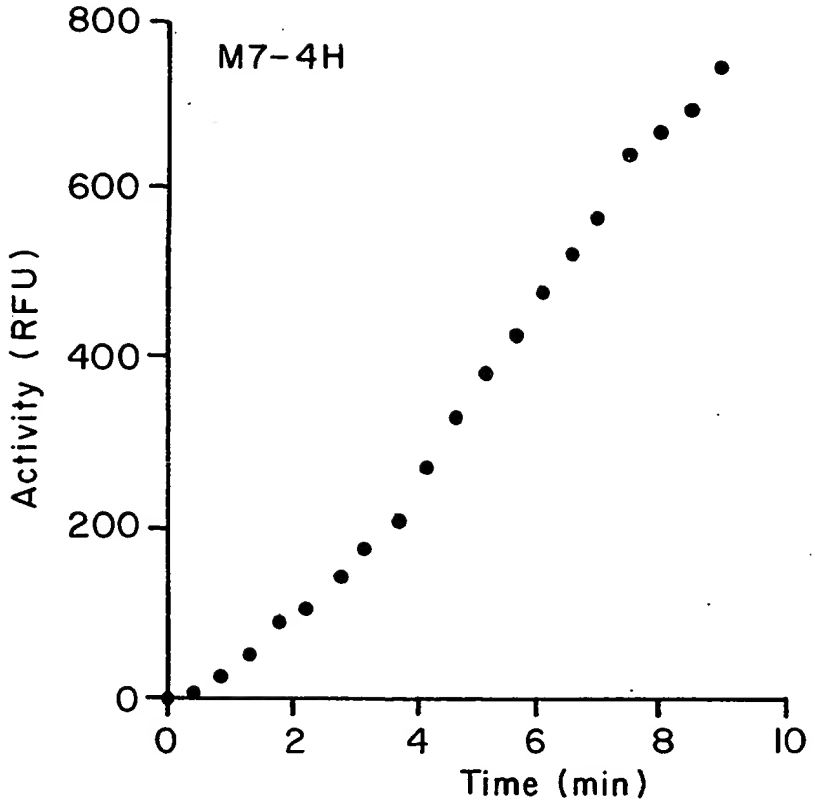


FIG. 19D



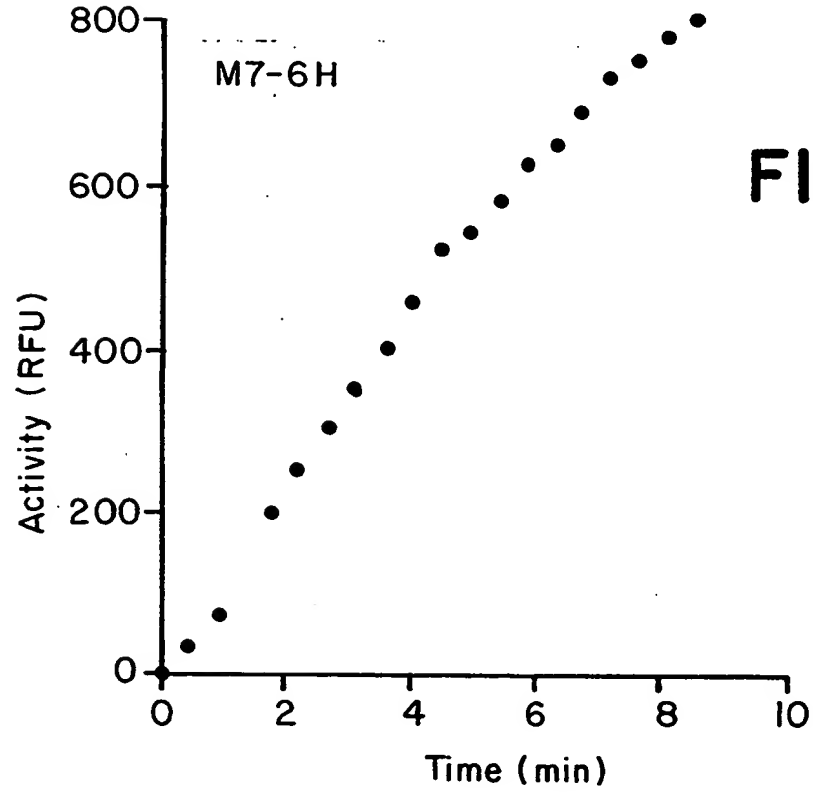


FIG. 19E

FIG. 19F

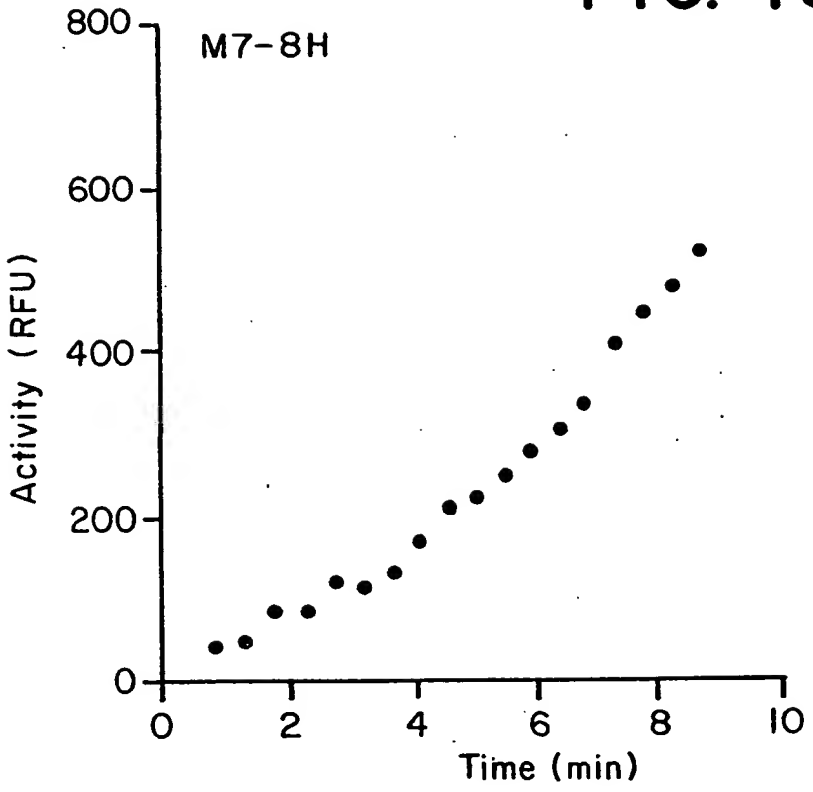


FIG. 20

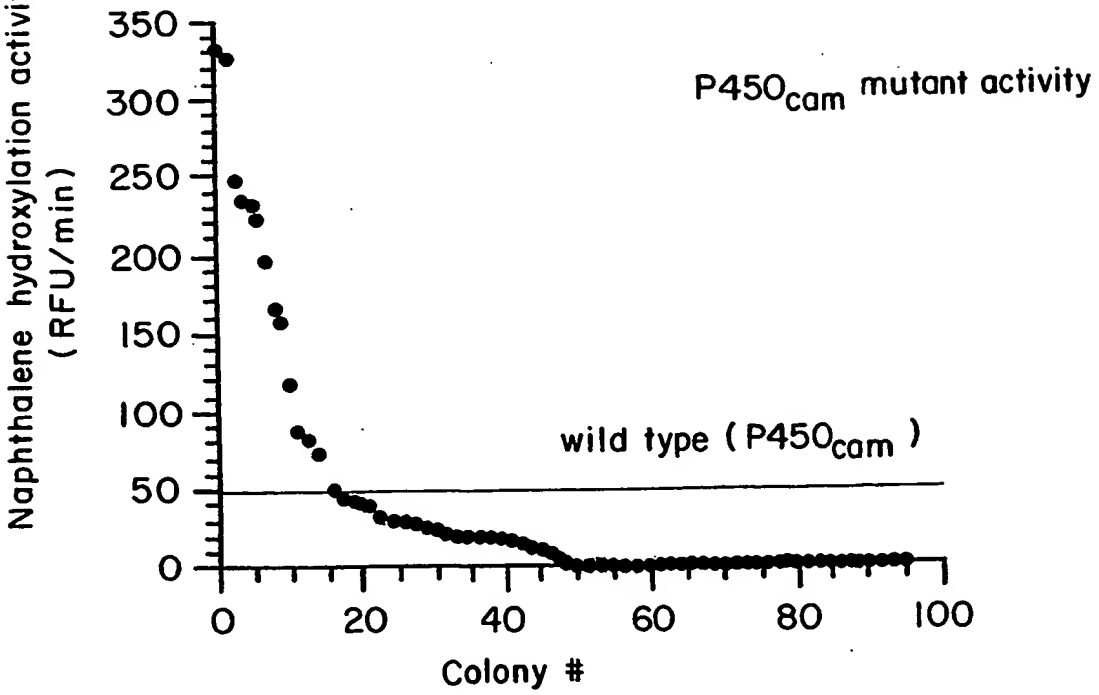


FIG. 22

ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA CCA GCC ATG GCC
Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala Met Ala

FIG. 23A

```
          10         20         30         40         50         60
ATGCAGTTAACCCCTACATTCTACGACAATAGCTGTCCCAACGTGTCCAACATCGTTCGC
 M  Q  L  T  P  T  F  Y  D  N  S  C  P  N  V  S  N  I  V  R

          70         80         90        100        110        120
GACACAATCGTCAACGAGCTCAGATCCGATCCCAGGATCGCTGCTTCAATATTACGTCTG
 D  T  I  V  N  E  L  R  S  D  P  R  I  A  A  S  I  L  R  L

         130        140        150        160        170        180
CACTTCCATGACTGCTTCGTGAATGGTTGCGACGCTAGCATATTACTGGACAACACCACC
 H  F  H  D  C  F  V  N  G  C  D  A  S  I  L  L  D  N  T  T

         190        200        210        220        230        240
AGTTTCCGCACTGAAAAGGATGCATTCGGGAACGCTAACAGCGCCAGGGGCTTTCCAGTG
 S  F  R  T  E  K  D  A  F  G  N  A  N  S  A  R  G  F  P  V
```

A——— – ——————————————————— – ———A

FIG. 23B

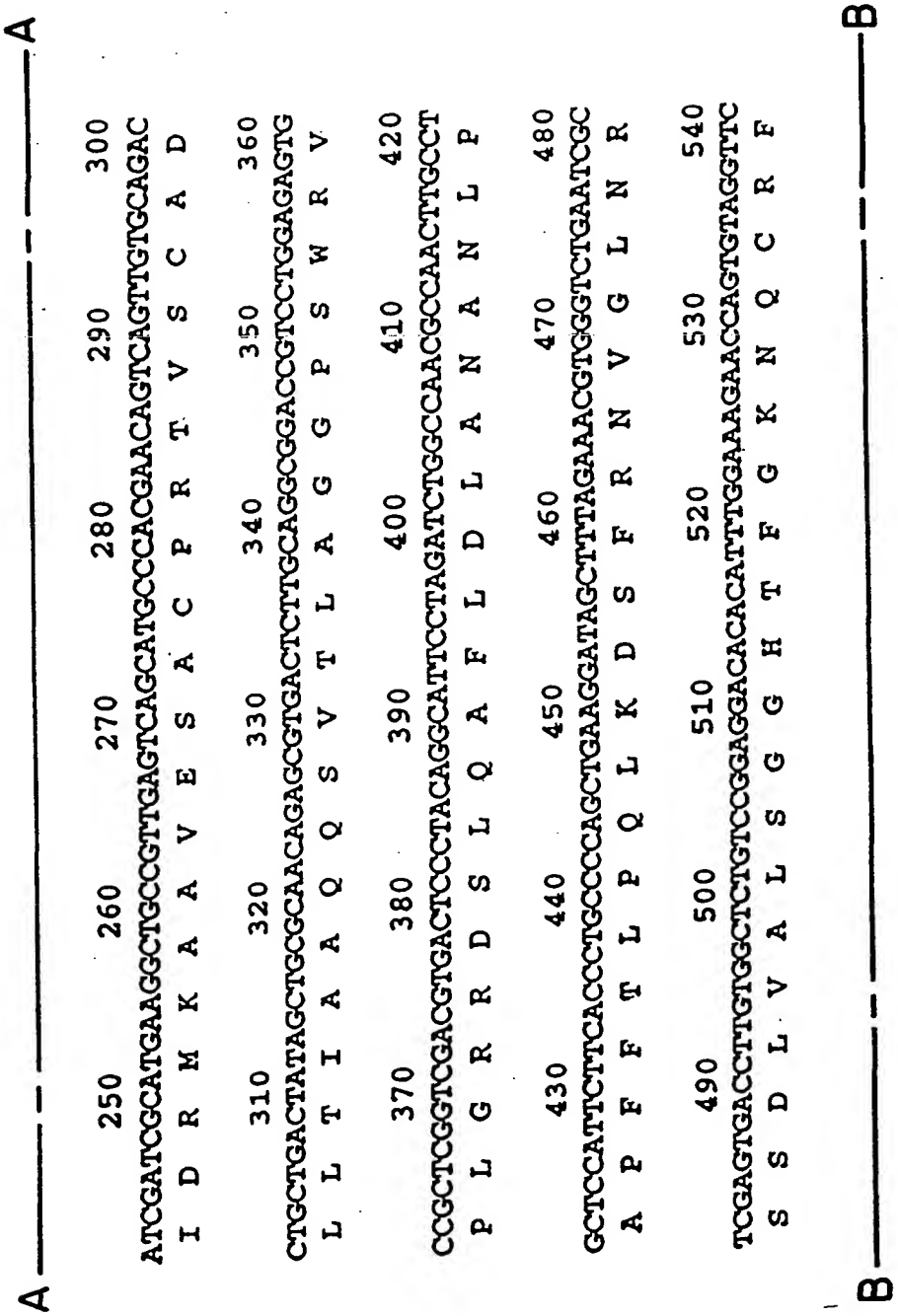

A
A
250 260 270 280 290 300
ATCGATCGCATGAAGGCTGCCGTTGAGTCAGCATGCCCACGAACAGTCAGTTGTGCAGAC
I D R M K A A V E S A C P R T V S C A D
310 320 330 340 350 360
CTGCTGACTATAGCTGCGCAACAGAGCGTGACTCTTGCAGGCGGACCGTCCTGGAGAGTG
L L T I A A Q Q S V T L A G G P S W R V
370 380 390 400 410 420
CCGCTCGGTCGACGTGACTCCCTACAGGCATTCCTAGATCTGGCCAACGCCAACTTGCCT
P L G R R D S L Q A F L D L A N A N L P
430 440 450 460 470 480
GCTCCATTCTTCACCCTGCCCCAGCTGAAGGATAGCTTTAGAAACGTGGGTCTGAATCGC
A P F F T L P Q L K D S F R N V G L N R
490 500 510 520 530 540
TCGAGTGACCTTGTGGCTCTGTCCGGAGGACACACATTTGGAAAGAACCAGTGTAGGTTC
S S D L V A L S G G H T F G K N Q C R F
B
B

FIG. 23C

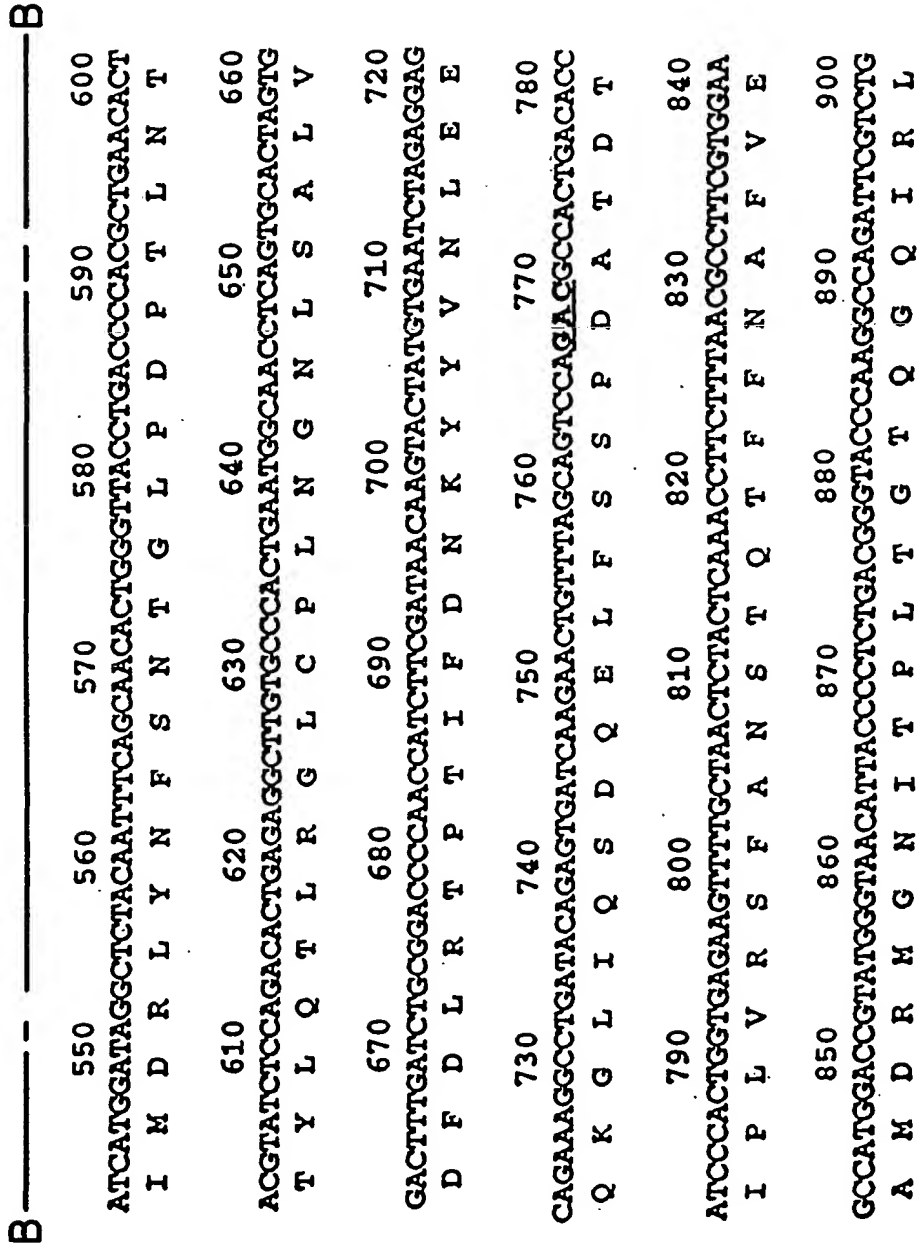

B
B
550 560 570 580 590 600
ATCATGGATAGGCTCTACAATTTCAGCAACACTGGGTTACCTGACCCACGCTGAACACT
I M D R L Y N F S N T G L P D P T L N T
610 620 630 640 650 660
ACGTATCTCCAGACACTGAGAGGCTTGTGCCCACTGAATGGCAACCTCAGTGCACTAGTG
T Y L Q T L R G L C P L N G N L S A L V
670 680 690 700 710 720
GACTTTGATCTGCGGACCCCAACCATCTTCGATAACAAGTACTATGTGAATCTAGAGGAG
D F D L R T P T I F D N K Y Y V N L E E
730 740 750 760 770 780
CAGAAAGGCCTGATACAGAGTGATCAAGAACTGTTTAGCAGTCCAGACGCCACTGACACC
Q K G L I Q S D Q E L F S S P D A T D T
790 800 810 820 830 840
ATCCCACTGGTGAGAAGTTTTGCTAACTCTACTCAAACCTTCTTTAACGCCTTCGTGGAA
I P L V R S F A N S T Q T F F N A F V E
850 860 870 880 890 900
GCCATGGACCGTATGGGTAACATTACCCCTCTGACGGGTACCCAAGGCCAGATTCGTCTG
A M D R M G N I T P L T G T Q G Q I R L
910 920 930
AACTGCAGAGTGGTCAACAGCAACTCT
N C R V V N S N S

FIG. 24

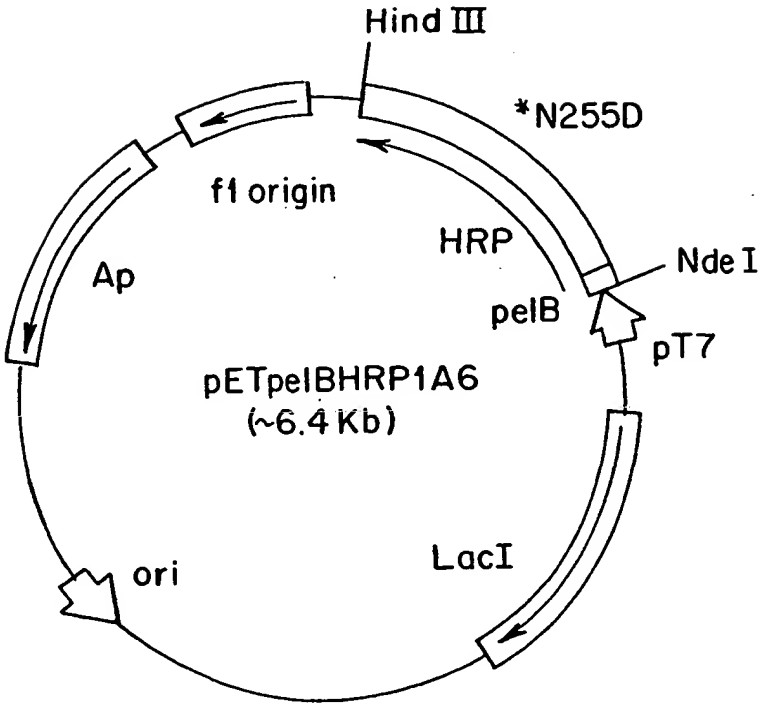
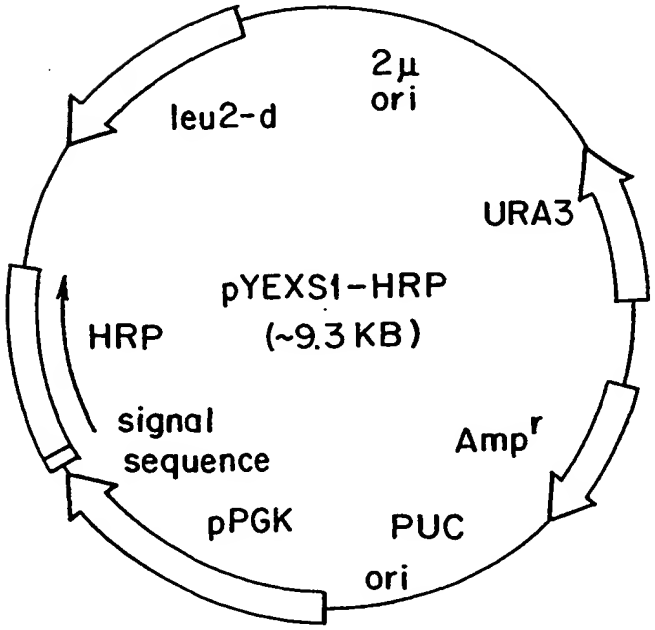

Hind III
*N255D
f1 origin
HRP
Nde I
Ap
pelB
pT7
pETpelBHRP1A6
(~6.4 Kb)
ori
LacI

FIG. 25


2μ
ori
leu2-d
URA3
pYEXS1-HRP
(~9.3 KB)
HRP
signal
sequence
Amp^r
pPGK
PUC
ori

INTERNATIONAL SEARCH REPORT

ional Application No
PCT/US 00/28768

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/26 C12Q1/28 C12N15/81 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 60096 A (JOO HYUN ;LIN ZHANGLIN (US); ARNOLD FRANCES H (US); CALIFORNIA INS) 25 November 1999 (1999-11-25) the whole document	1-145
X	JOO H ET AL: "A HIGH-THROUGHPUT DIGITAL IMAGING SCREEN FOR THE DISCOVERY AND DIRECTED EVOLUTION OF OXYGENASES" CHEMISTRY AND BIOLOGY,GB,CURRENT BIOLOGY, LONDON, vol. 6, no. 10, 10 September 1999 (1999-09-10), pages 699-706, XP000892823 ISSN: 1074-5521 the whole document	1-145

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 2001	13/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Tuynman, A

Form PCT/ISA/210 (second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 107-114 and 131 relate to an oxygenase enzyme variant defined by reference to a desirable characteristic or property, namely being obtainable by one of the methods of claims 1, 20, 47, 58, 65, 88, 103, 104 or 126. Present claims 123-125, relate to an oxygenase enzyme variant defined by reference to a desirable characteristic or property, namely being a sequence-conservative variant of claim 122, a function conservative variant of claim 122, or a variant encoded by a first polynucleotide that hybridizes to a second polypeptide encoded by an enzyme of claim 122 under high stringency conditions. Present claims 130,133-136, relate to an oxygenase enzyme variant defined by reference to a desirable characteristic or property, namely having an improved enzyme activity or stability in comparison with a provided P450 enzyme.

The claims cover all oxygenase enzyme variants having these characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such oxygenase enzyme variants. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the oxygenase enzyme variants by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the oxygenase enzyme variants mentioned in example 8, page 59, line 10-page 60, line 24.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

ional Application No
PCT/US 00/28768

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9960096 A	25-11-1999	AU 4200999 A	06-12-1999
		AU 5134599 A	21-02-2000
		EP 1100891 A	23-05-2001
		WO 0006718 A	10-02-2000

Form PCT/ISA/210 (patent family annex) (July 1992)